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THE ROLE OF HUMAN NOROVIRUS SURROGATES, FELINE CALICIVIRUS AND MURINE NOROVIRUS, ON NON-POROUS AND SOFT POROUS SURFACES

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ABSTRACT

Human Noroviruses (HuNoV) are the leading cause of acute gastroenteritis worldwide as well as the leading cause of foodborne disease in the U.S. HuNoV can persist in the environment even after proper disinfection, making preventing HuNoV infections and controlling subsequent outbreaks extremely challenging. Epidemiological evidence suggests that soft surfaces may be a relevant source of HuNoV due to the inability to effectively decontaminate. The objectives of this study were to: 1) review the current published literature on prevalence, transmission, and disinfection pertaining to HuNoV and surrogates with an emphasis on soft surfaces as fomites, 2) optimize a recovery method capable of efficiently recovering microorganisms from cotton fabric, and 3) develop a method for assessing the recovery and disinfection of viruses on soft surfaces using two HuNoV surrogates, Feline Calicivirus (FCV) and Murine Norovirus (MNV).

In order to determine the most efficient method for recovery of microorganisms from soft surfaces we evaluated the recovery efficiency (RE) of *Escherichia coli* from cotton swatches using three elution-agitation methods. We found that RE using stomaching, sonication, and vortexing was not significantly different ($p>0.05$), resulting in approximately 21-30% RE. The most efficient method of recovery was achieved using a combination of sonication for 5 min at 40 kHz prior to stomaching for 5 min at 260 rpm. This resulted in a RE of 65% of *E. coli* dried on cotton swatches.

To evaluate our proposed method for testing disinfectants against HuNoV on soft surfaces, we compared the recovery efficiency and disinfection efficacy of FCV and MNV bound to glass, polyester, and cotton. FCV and MNV were recovered from glass, cotton and polyester at 35.22, 5.59, 0.15% and 24.27, 14.69, and 0.85%, respectively. Two sanitizers, bleach (5,000 ppm NaOCl) and Oxivir (2,656 ppm H_2O_2) were able to inactivate FCV (2.5-4.7 reduction) below the limit of detection on all 3 surface types. Only bleach was able to inactivate MNV (2.2-3.8 log reduction) below the limit of detection on all 3 surface types. Inactivation of MNV by Oxivir resulted in a reduction of 1.3, 0.57, and 0.17 log pfu/ml on glass, polyester, and cotton, respectively. Reduction of viral RNA measured by RT-qPCR using bleach resulted in 2.72-4.06 log reduction for FCV and 2.07-3.04 log reduction for MNV on all 3 surface types. Reduction of viral RNA by Oxivir resulted in 1.89-3.4 log reduction for FCV and 0.54-0.85 log reduction for MNV. We found that the virus type had a significant ($p<0.001$) influence on the recovery and disinfection of soft surfaces. In addition we found that recovery was also significantly different from non-porous, synthetic porous, and natural porous surfaces ($P<0.05$).

The results of our study clearly indicate that both microorganism and surface type influence recovery efficiency and disinfection efficacy. Due to the low recovery observed on soft surfaces, further studies on recovery methods for soft surfaces are needed in order to document the 4 log reduction needed to establish virucidal efficacy. In addition we recommend that the use of FCV as a surrogate be carefully considered, as it may not be the most suitable surrogate for evaluating the efficacy of disinfectants against HuNoV.

DEDICATION

I would like to dedicate this work to my parents, Nella Allen and Dewey and Despina Yeargin, without whose support and encouragement this would not have been possible.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Xiuping Jiang for her patience and support throughout this process. I would like to thank Dr. Angela Fraser, Dr. Tamara McNealy, and Dr. Charles Pettigrew for all of their contributions and for serving on my thesis committee. My research is based upon work supported by the National Institute of Food and Agriculture, USDA, under Agreement No. 2011-68003-30395.

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CHAPTER ONE

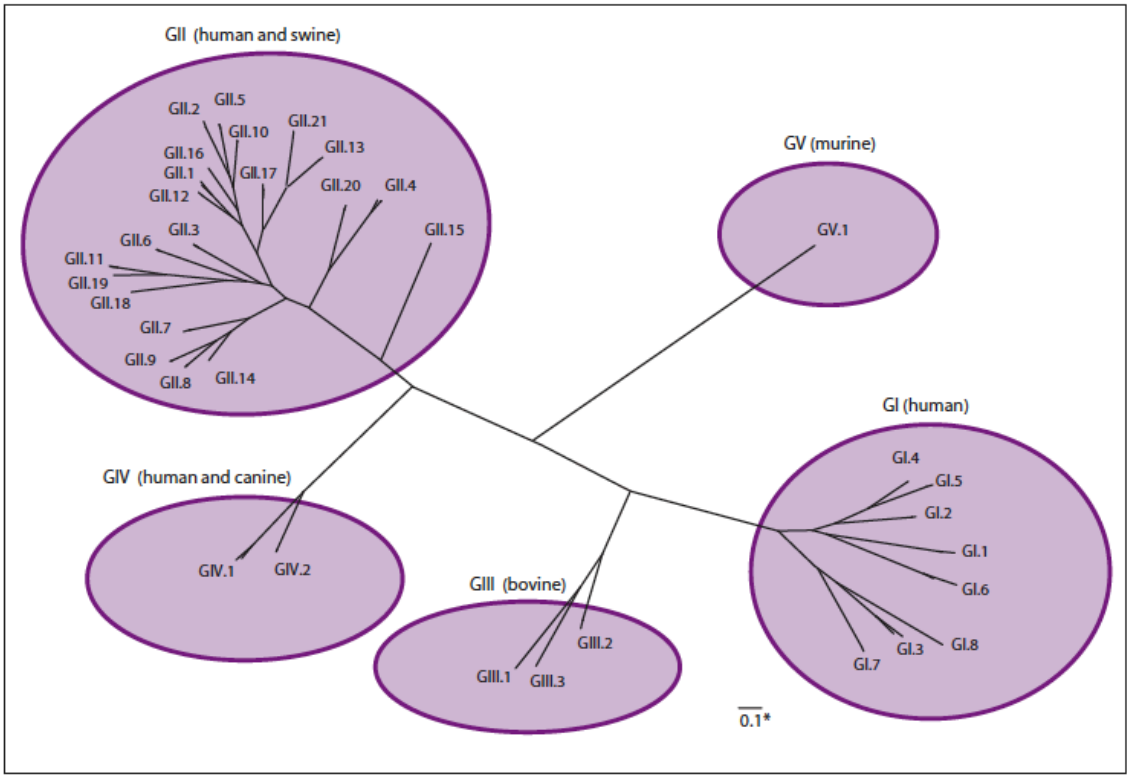
LITERATURE REVIEW

Introduction

Foodborne diseasedisease is a major problem worldwide, affecting both producers and consumers of food. Surveillance data of foodborne disease in the U.S. indicates that each year there are 48 million cases of foodborne disease resulting in 125,000 hospitalizations and 3,000 deaths. The 2011 estimates of foodborne diseaseshowed that Human Norovirus (HuNoV) was the leading cause of foodborne disease caused by known pathogens, and one of the top 3 leading causes of death (Scallan et al. 2011). Additionally HuNoV is the leading cause of acute gastroenteritis in the United States (Hall et al. 2013; Kosa et al. 2013).

HuNoV is a small round viral particle ranging from 27-30 nm, first identified following an outbreak of gastroenteritis in Norwalk, Ohio in 1972 (Marks et al. 2000). HuNoV belongs to the *Caliciviridae* family and is classified into 5 genogroups numbered I-V based on phylogenetic analysis of the Open Reading Frame 2 (ORF2) (Morillo et al. 2011). These groups are further divided into more than 35 genotypes. Of the 5 genogroups, GI, GII, and GIV are known to cause illness in humans with GII type 4 being responsible for 70% of HuNoV outbreaks (**Figure 1.1**) (Morillo et al. 2011).

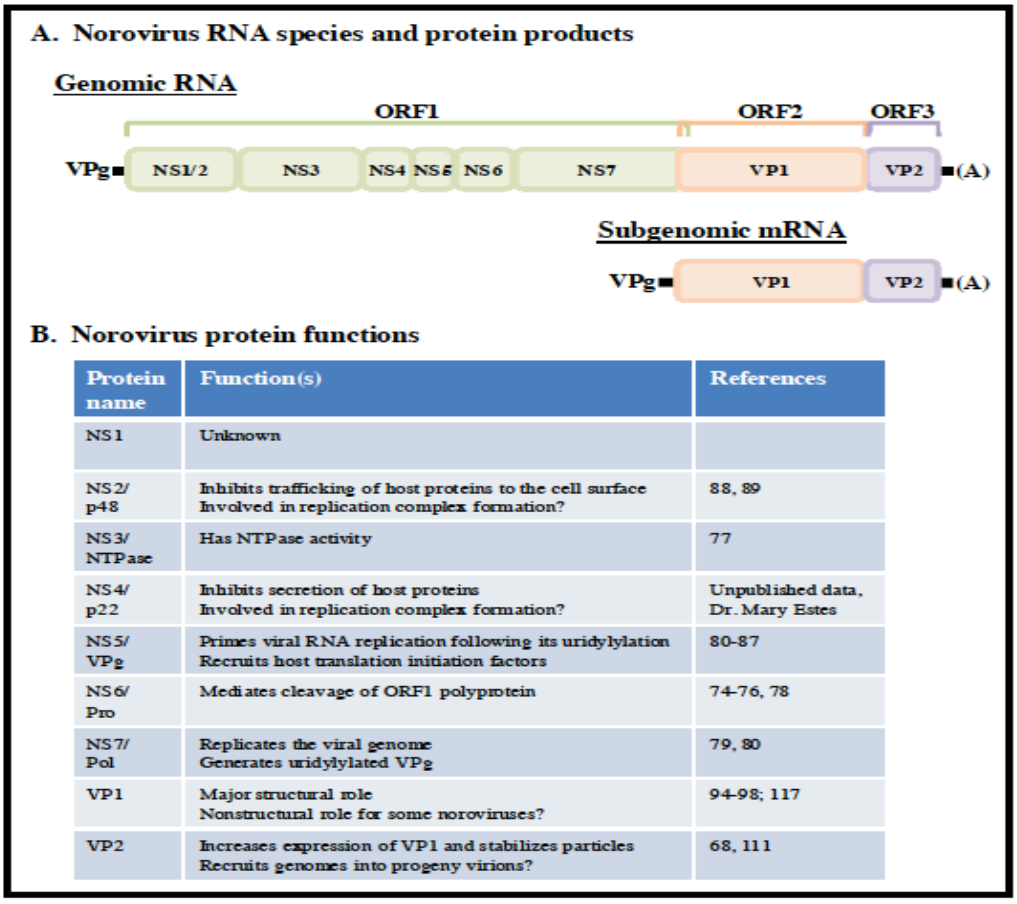
Figure 1.1 Classification of Noroviruses into 5 genogroups and 35 genotypes



Sources: Data from Zheng DP, Ando T, Fankhauser RL, Beard RS, Glass RI, Monroe SS. Norovirus classification and proposed strain nomenclature. Virology 2006;346:312–23; Wang QH, Han MG, Cheetham S, Souza M, Funk JA, Saif LJ. Porcine noroviruses related to human noroviruses. Emerg Infect Dis 2005;11:1874–81; CDC, unpublished data, 2011; graphic developed by Everardo Vega, PhD, CDC.
* The scale bar of 0.1 reflects the number of amino acid substitutions per site.

Noroviruses are non-enveloped and as such consist solely of a capsid and nucleic acid. The nucleic acid is plus-sense single strand RNA that makes up the viral genome consisting of approximately 7.5 kb (Morillo et al. 2011). The genome contains three open reading frames (**Figure 1.2**). The first open reading frame (ORF1) encodes for several proteins including the RNA dependent RNA polymerase. Open reading frame 2 (ORF2) encodes a capsid protein that plays a major role in viral replication. The third open reading frame (ORF3) encodes for a protein that interacts with genome RNA to form the virion (Morillo et al. 2011).

Figure 1.2 HuNoV genomes and its protein functions



B. Norovirus protein functions

Protein name	Function(s)	References
NS1	Unknown	
NS2/ p48	Inhibits trafficking of host proteins to the cell surface Involved in replication complex formation?	88, 89
NS3/ NTPase	Has NTPase activity	77
NS4/ p22	Inhibits secretion of host proteins Involved in replication complex formation?	Unpublished data, Dr. Mary Estes
NS5/ VPg	Primes viral RNA replication following its uridylylation Recruits host translation initiation factors	80-87
NS6/ Pro	Mediates cleavage of ORF1 polyprotein	74-76, 78
NS7/ Pol	Replicates the viral genome Generates uridylylated VPg	79, 80
VP1	Major structural role Nonstructural role for some noroviruses?	94-98; 117
VP2	Increases expression of VP1 and stabilizes particles Recruits genomes into progeny virions?	68, 111

(Karst et al. 2010) – Is this the original source?

Primary transmission of HuNoV occurs through person-to-person contact (66%) and consumption or handling of contaminated food (26%) (Kosa et al. 2013). Secondary transmission via the environment, however, has been shown to be an important route of transmission, often responsible for prolonged and reoccurring outbreaks (Lopman et al. 2012). Outbreaks of HuNoV are often associated with high traffic indoor environments, such as schools, nursing homes, catered events, and cruise ships. Environmental transmission of HuNoV depends upon many factors including virus viability and resistance to disinfection, environmental conditions, as well as the surface contaminated.

Studies have demonstrated the ability of HuNoV RNA to persist on surfaces for up to 42 days (Liu et al. 2009; Escudero et al. 2012). Currently, the most effective way to inactivate HuNoV in the environment is cleaning with 1,000-5,000 ppm bleach (Barker et al. 2004; Hall et al. 2011). However, this recommendation applies only to hard, non-porous surfaces. The use of bleach on soft surfaces is not recommended outside of laundering practices because it can cause damaging effects to the surface. Epidemiological evidence has attributedseveral outbreaks of HuNoV to soft surfaces due to ineffective decontamination of the surfaces (Chessbrough et al.1997; Chessbrough et al. 2000; Evans et al. 2002).

Soft surfaces are inherently difficult to decontaminate because of the complexity of their physical structure. Differences in fiber composition and fabrication can influence the efficacy of chemical disinfectants, which makes it difficult to identify a cleaning agent or method that is applicable to all soft surface types. In order to effectively kill HuNoV on soft surfaces, virucidal testing methods need to be developed and applied to an environmental disinfection protocol. The U.S. Environmental Protection Agency (EPA) currently registers disinfectants against HuNoV for use on hard non-porous surfaces only (EPA. 2009). The methods for testing these disinfectants follow the EPA DIS/TSS-7 guidelines for virucidal efficacy testing (EPA. 1981). The only current method for soft surface testing is an adaption of the American Society for Testing and Materials (ASTM) method for efficacy testing of sanitizers on inanimate non-food-contact surfaces, however, this method does not include viral testing (EPA 2012). The following literature review will discuss factors that contribute to the role of HuNoV as a

pathogen, including environmental persistence, multiple routes of transmission, and resistance to disinfection.

HuNoV Strain Variation

As shown in Figure 1.1, genogroups GI and GII are primarily associated with human illness, with GII.4 associated with most cases. HuNoV belonging to the GII.4 are of special concern as they are associated with higher rates of hospitalization and death (Barclay et al. 2013). Since 1995, a new strain of GII.4 has emerged every 2-3 years. Phylogenetic analysis shows that strain variations in GII.4 are caused by amino acid changes in the epitopes of the P2 domain (van Beek et al. 2012). The emergence of new strains can be, but is not always associated with an increase in outbreaks. For example, when the previously dominant strain GII.4 New Orleans emerged, surveillance data revealed there was no significant increase in the number of outbreaks as compared to previous data (Barclay et al. 2013). **Table 1.1** lists the dominant strains of HuNoV since 1995 and their association with outbreaks. In the past year there has been an increase in outbreaks related to a new strain designated GII.4 Sydney in several countries. In 2012, the United Kingdom experienced an early onset of HuNoV season with a 64% increase in the number of HuNoV outbreaks in which GII.4 Sydney was the dominant strain (Barclay et al. 2013). During September-December of 2012, 53% of reported cases of HuNoV in the U.S. were attributed to GII.4 Sydney. The number of cases increased significantly as the HuNoV season progressed. It cannot yet be confirmed that the increase in outbreaks is due strictly to the emergence of GII.4 Sydney, however, it has replaced its predecessor

GII.4 New Orleans as the most dominant strain responsible for human illness (van Beek et al. 2012).

Table 1.1 Emerging strains of HuNoV since 1995

Emergent GII.4 variant	Yrs of circulation	Epidemic Season	Other name(s)
95/96-US	1995–2002	1995–1996	Grimsby
Farmington Hills	2002–2005	2002–2003	2002 variant
Hunter	2003–2006	None	2004 variant
Yerseke	2006–2008	2006–2007	Laurens, Nijmegen, 2006a variant
Den Haag	2006–present	2006–2007	Minerva, 2006b variant
New Orleans	2009–present	None*	

Sources: Zheng DP, Widdowson MA, Glass RI, Vinje J. Molecular epidemiology of genogroup II-genotype 4 noroviruses in the United States between 1994 and 2006; J Clin Microbiol 2010;48:168–77; CDC, unpublished data, 2010.

* On the basis of data available as of September 2010, the New Orleans GII.4 variant has not been associated with an increased number of norovirus outbreaks in the United States.

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Pathogenesis

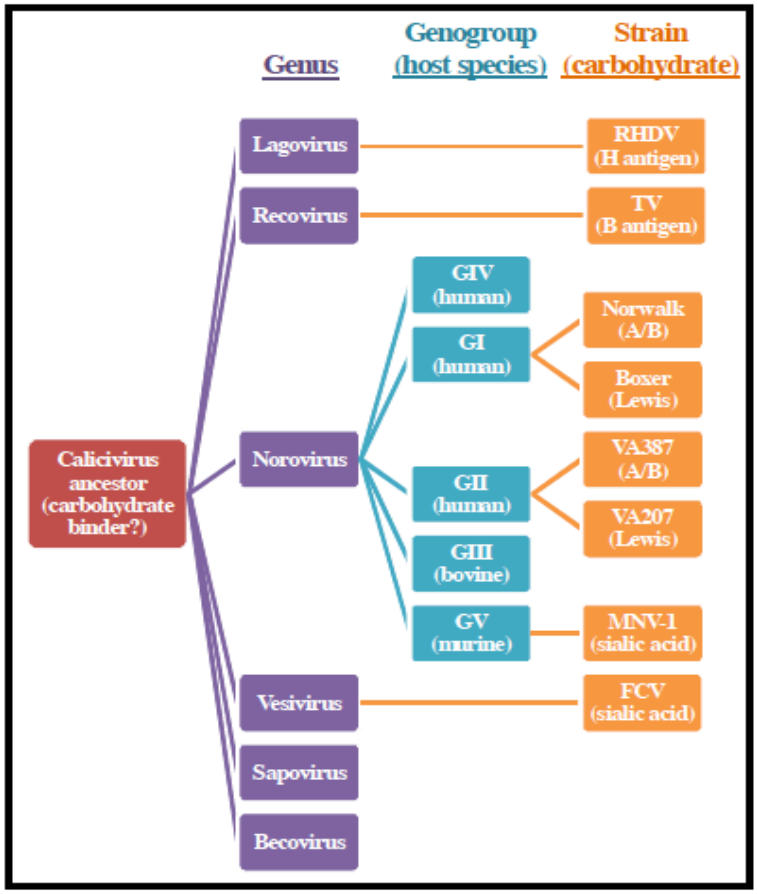
Because of the lack of cell culture system to study HuNoV, there is a lack of information as to the exact mechanism of infection. Most available knowledge comes from studying human volunteers who have been previously infected. Infections of HuNoV occur through the fecal-oral route due to consumption of contaminated food or water, through the environment, or person-to-person. The infectious dose for HuNoV is very low, estimated to be about 10-100 viral particles (Karst et al. 2010; Marks et al. 2000). As a non-enveloped virus HuNoV is resistant to low pH, which allows it to pass through the stomach unaffected. Once ingested, the pathogen binds to the histo-blood group antigens (HGBA) expressed on mucosal epithelial cell surfaces, which induces histological changes to the intestinal mucosa resulting in gastrointestinal illness (Karst et al. 2010). Replication of the virus occurs in the enterocyte cytoplasm, where the viral RNA serves as mRNA for protein synthesis (Morillo et al. 2011). Symptoms of illness include vomiting and abdominal cramps as well as diarrhea and occasionally a low-grade

fever. Projectile vomiting is the most common symptom associated with illness. The illness is typically self-limiting and resolves itself within 24-72 h,, however, the pathogen is shed in feces for up to 2 weeks, and sometimes longer (Marks et al. 2000; Rabenau et al. 2003). In immunocompromised individuals or other high-risk groups, such as infants and the elderly, HuNoV has a potential to lead to long-term illness and even death, usually due to dehydration.

Role of Surrogates

A major hinderance in the study of HuNoV is the lack of culturability in cell lines and animal models. As a result surrogates for HuNoV have been used to study the susceptibility to decontamination methods and environmental persistence. The most widely used surrogates are the animal models Feline Calicivirus (FCV) and Murine Norovirus (MNV-1). **Figure 1.3** shows the relationship between HuNoV and the most popular surrogates. While these two surrogates are useful in predicting how HuNoV will respond to certain stresses, they pose certain limitations. FCV is easy to propagate but is known to be less stable at low pH. The ability to survive at low pH is a major resistance mechanism for HuNoV allowing it to survive the environment of the stomach (Girard et al. 2010). MNV-1 is a better candidate, as it is more resistant than FCV to pH and organic solvents (Poschetto et al. 2007). But despite this similarity, MNV-1 has been shown to be less resistant to certain disinfectants than HuNoV, showing 1-2 logs more reduction in viral titer by bleach than what has been observed with HuNoV (Girard et al. 2010).

Figure 1.3 Summary of Caliciviruses



(Karst et al. 2010)

Another possible surrogate is the male-specific bacteriophage MS2. MS2 is similar in shape and size to HuNoV and is also a single-stranded RNA virus (Hirneisen et al. 2010). In addition MS2 is adapted to the intestinal tract and has been shown to be a successful indicator for HuNoV in experimental oyster contamination (Dore et al. 2000). MS2 is often used as an internal control to validate recovery, extraction, and detection methods for HuNoV (Mormann et al. 2010). Bacteriophages are useful as surrogates as

they are relatively easy to propagate and require a shorter incubation period for detection than virus-plaque assays do.

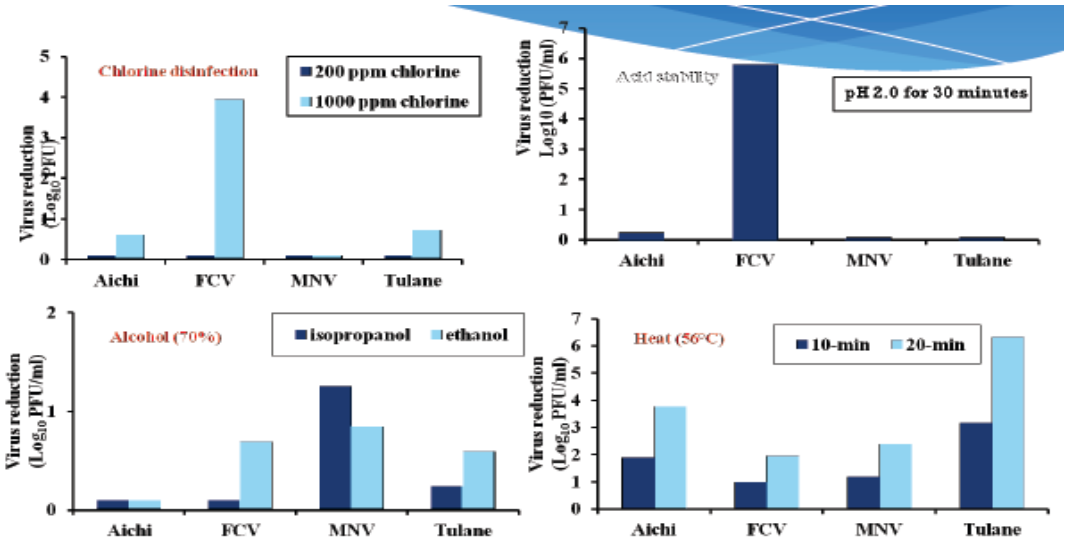
Recently there have been two more animal virus surrogates and one human virus surrogate developed for the study of HuNoV. These viruses are the Porcine Sapovirus (SaV), Tulane Virus (TV), and Aichi Virus (AV). Each model has its own distinct advantages and disadvantages; however, they have yet to be fully evaluated (Li et al. 2012). The most noteworthy aspect of SaV is that it causes gastroenteritis in infected pigs. TV does not cause gastroenteritis in its host, however, it shares another important quality with HuNoV because TV grown in cell culture has been demonstrated to bind to an HBGA similar to what HuNoV binds to in humans. Aichi virus is an especially interesting surrogate, as it is the only human model available to study HuNoV.

These surrogates have been evaluated for not only their pathogenic similarity to HuNoV but also their response to physiochemical parameters. As shown in **Figure 1.4**, Cromeans et al. (2013) reported the response of surrogates FCV, MNV, AV, and TV to chlorine, pH, heat, and alcohols. When exposed to 200 ppm chlorine, all 4 viruses had less than 1 log reduction in viral titer. FCV was shown to be more susceptible to chlorine than other surrogates, displaying a >3 log reduction when exposed to 1,000 ppm chlorine, whereas other surrogate viruses demonstrated <1 log reduction in viral titer. FCV was also shown to be the most susceptible to pH showing >6 log reduction in viral titer at pH 2 for 20 min. Other surrogates again also demonstrated <1 log reduction in viral titer under the same conditions. These results agree with other studies that show FCV as a less resistant surrogate. Both TV and AV were more susceptible than the other surrogates to

heat treatment showing a >3 and >6 log reduction after 20 min at 56 **°C** respectively, as compared to 2-3 log reduction in viral titer for MNV and FCV. MNV was the most susceptible to alcohol demonstrating a >1 log reduction in viral titer when exposed to 70% isopropanol for 1 min. Wang et al. (2012) studied SaV and reported the virus was resistant to pH ranging from 4-8 and had less than 1 log reduction in viral titer at pH 3. SaV demonstrated similar resistance as MNV to heat and chlorine but was more resistant than FCV. SaV was also more resistant to UV than either FCV or MNV. While the best surrogate for the study of HuNoV has not yet been determined, current data suggest that TV and especially FCV may not be the best surrogates for studying persistence and disinfection (Hirneisen et al. 2013).

FCV is currently the only EPA approved surrogate for testing the efficacy of disinfectants against HuNoV. The main issue with this is there have been numerous studies proving that FCV is more sucseptible to inactivation than is HuNoV. Because of this, there may be currently registered disinfectants that could be ineffective against HuNoV. Despite the overwhelming evidence against the use of FCV, there has yet to be a new approved surrogate.

Figure 1.4 Physiochemical response of 4 HuNoV surrogates – Should the first letter be capitalized – it is on other figure headers



(Cromeans et al. 2013)

Routes of Transmission

Outbreaks of HuNoV are most often associated with health-care facilities (35.4%), restaurants and catered events (31.1%), cruise ships (20.5%), and schools or communities (13%) (Hall et al. 2013; Zheng et al. 2010). There are several exposure pathways for HuNoV including person-to-person (66%), consumption of contaminated food (25%) or water (0.2%), and environmental transmission (0.3%) (Hall et al. 2013). Symptoms of the illness, such as diarrhea and vomiting, facilitate the spread of the virus further as there can be 10^5-10^9 HuNoV particles/g of stool and approximately 10^7 virus particles shed during an episode of vomiting. These particles can be deposited into the environment through direct contact with bodily fluids or through the generation of aerosols (Marks et al. 2000; Morillo et al. 2011).

Person-to-person transmission is the leading cause of HuNoV infections, however, secondary contamination from environmental fomites is often what leads to ongoing or reoccurring outbreaks. Laboratory studies have shown multiple ways that HuNoV can be transmitted through the environment. Barker et al. (2004) observed the deposit of HuNoV from contaminated fingers to hard non-porous surfaces and the transfer of HuNoV from a contaminated surface by clean fingers. When fingers were contaminated with HuNoV contained in feces and allowed to dry for 15 sec, researchers reported that up to 7 surfaces could be contaminated with HuNoV as measured by RT-qPCR (Barker et al. 2004) Out of 8 surfaces touched, the first 4 were contaminated in 100% of trials, surfaces 5 and 6 were contaminated in 75% of trials, surface 7 was contaminated in 25% of the trials, and surface 8 remained negative in all four trials. Barker et al. (2004) further studied the transfer of HuNoV contained in feces by clean fingers. Melamine surfaces contaminated with HuNoV and allowed to dry for 15 min after which clean fingers were then used to touch the contaminated surface followed by three additional fomites. HuNoV was transferred from the contaminated surface by clean fingers to 4 out of 10 door handles, 5 out of 10 telephone receivers, and 3 out of 10 taps. D'Souza et al. (2010) evaluated the transfer of HuNoV and FCV from artificially contaminated stainless steel to lettuce leaves under wet and dry conditions. Both viruses were deposited onto stainless steel and allowed to dry for 10, 30, or 60 minutes. Transfer of HuNoV was considered successful if a signal was detected by RT-PCR whereas transfer for FCV was evaluated via plaque assay. After all three dry times, HuNoV was transferred to wet lettuce. When dry lettuce was used, ,however, HuNoV could only be

transferred after 10 minutes of drying time. When FCV was allowed to dry for 0,10, 30, and 60 min the observed rate of transfer to wet lettuce was significantly different from time 0 to 60 min showing a decline from an initial 6.79% transfer rate to 4.27% after 60 min of drying. When dry lettuce was used, the transfer observed at time 0 was significantly different from that observed at time 10, 30, and 60 min exhibiting a decrease in transfer rate from an initial 4.93% at time 0 to 0.24% at time 60. The ability of HuNoV and FCV to be transferred with greater ease between two wet surfaces and at decreased drying time was attributed to the increased moisture content facilitating the transfer.

Persistence of HuNoV

The ability of HuNoV to be transferred via the environment is largely due to its ability to persist for an extended period of time under a variety of conditions. As a non-enveloped virus, HuNoV has increased resistance to environmental stresses like temperature, humidity, UV and pH (Girard et al. 2010; Hall et al. 2011). Multiple studies have shown that HuNoV RNA can be detected on hard non-porous surfaces by RT-PCR up to 42 days after inoculation when held under ambient conditions (temperature, relative humidity, and light) (Escudero et al. 2012; Liu et al. 2009). At low temperatures (4-7 **°C**) HuNoV easily survived for 14 days on food surfaces showing little reduction in viral RNA (Bae and Shwab. 2008; Escudero et al. 2012). In order to determine whether HuNoV remains infectious during environmental persistence, HuNoV RNA has been treated with Proteinase K and RNase (Lamhoujeb et al. 2008; Lamhoujeb et al. 2009). Using enzymatic pretreatment, infectious HuNoV has been found to persist on lettuce and

turkey for up to 10 days at 7 **°C** (Lamhoujeb et al. 2008). Using the same enzymatic pretreatment technique, HuNoV persistence has also been evaluated on food-contact surfaces. HuNoV remained infectious on stainless steel and PVC for 56 and 49 days, respectively, at 7 **°C**. At 20 **°C** HuNoV has been shown to remain infectious for 7 and 28 days on both surfaces under low (30%) and high (86%) RH conditions, respectively (Lamhoujeb et al. 2009). Surrogate studies have also shown extended persistence with FCV and MNV remaining infectious 28 and 20 days, respectively, when dried on a stainless steel surface at room temperature. Additionally FCV has displayed extended survival of up to 56 days at 4 **°C** (Doultree et al. 1999; Liu et al. 2009). A list of the persistence of HuNoV on environmental surfaces is in **Table 1.2**.

Table 1.2 Environmental persistence of HuNoV and surrogates

Surface/Medium	Virus	Treatment	Significant Results	Reference
Surface water Ground water	FCV MNV-1 HuNoV. GI	4 and 25 °C	Viruses were able to persist for 3-4 weeks Reduction in infectious titer was more significant at 25 °C than 4 °C FCV reduction was 0.08 log/day MNV and Nv.GI were 0.04 log/day	Bae and Shwab (2008)
Fecal suspensions dried onto stainless steel	FCV MNV-1	4 °C, 54% RH 25 °C, 75-85% RH	FCV and MNV remained infectious for at least 5 days under dry conditions and 7 days under wet, similar inactivation rates at 4 °C. MNV was more stable at room temp than FCV	Cannon et al. (2006)
Computer mouse, keyboard, telephone wire, telephone receiver, telephone buttons, brass disk	FCV	Room temperature incubation for up to 144 h	FCV survived a maximum of 72 h on telephone buttons and receivers with a 90% reduction in titer happening on the first 4-24 h	Clay et al. (2005)
Glass coverslips Stainless steel Formica Ceramic	FCV FCV HuNoV.GI	4, 20 (room temp) and 37 °C Ambient temperature	FCV persisted 56, 28, and <1 day at 4 °C, RT, and 37 °C FCV and HuNoV.GI were detected on all three surfaces up to 7 days	Doultree et al. (1999) D'Souza et al. (2006)
Fecal suspensions on Stainless steel Formica Ceramic Lettuce	MNV-1 HuNoV.GI SMV (HuNoV.GII)	Surfaces were stored at ambient conditions for 42 days. Lettuce to 14 days, RT and 4 °C	HuNoV.GI and HuNoV.GII remained detectable up to 42 days on food prep surfaces and 14 days on lettuce at 4 °C and RT MNV remained infectious up to 20 days on food prep surfaces and 14 days on lettuce at 7 °C and RT	Escudero, et al. (2012)

Stainless steel Wood	MNV-1	Inactivation rates were determined for temperatures from 15-40 °C and RH 30-70% for 30 days	MNV survived longer on wood than steel MNV persisted for 14 days at 25 °C and 30 % RH before displaying 90% reduction in titer	Kim et al. (2012)
Turkey Lettuce	HuNoV.GII	7 °C for up to 10 days	Infectious HuNoV.GII persisted on both surfaces for up to 10 days	Lamhoujeb et al. (2008)
Stainless Steel PVC	HuNoV.GII	7 °C 20 °C at 30% and 86% RH 56 days	Infectious HuNoV.GII persisted 56 and 49 days on stainless steel and PVC, respectively, at 7 °C Infectious HuNoV.GII persisted 7 and 28 days at 30% and 86% RH, respectively, at 20 °C on both surfaces	Lamhoujeb et al. (2009)
20 % fecal suspensions on Stainless steel Formica Ceramic Human fingers	HuNoV.GI SMV (HuNoV.GII)	Non-porous surfaces were kept at ambient conditions up to 42 days Fingers were inoculated and assayed to 120 min	HuNoV.GI and HuNoV.GII was detected up to 21-28 days and 42 days, respectively, on non porous surfaces HuNoV.GI and GII remained stable for up to 120 min on hands with minimum drop in titer	Liu et al. (2009)
Fecal suspension Stainless steel Lettuce Strawberries Ham	FCV	7 day incubation at RT and refrigeration conditions	Infectious virus was recovered up to 7 days from lettuce, stainless steel, and ham and 5 days from strawberries at 4 °C and up to 7 days from ham and stainless steel, 3 days from lettuce, and 1 day from strawberries at RT	Mattison et al. (2007)

Theoretical Aspects of Virus Adsorption

In order to inactivate viruses on environmental surfaces, a better understanding of how viruses attach to the surface is critical. Both chemical and physical forces influence adsorption of a virus to a surface, however, factors affecting the physical adsorption are more significant when dealing with virus and surface interactions (Gerba 1984). The physical adsorption of a virus is characterized as a displacement process where in a molecule of liquid on a surface is displaced by virus resulting in the formation of a single monolayer of virus. The amount of virus that can be absorbed is influenced by how much solute can be absorbed per gram of solid. Using isothermal models the amount of absorbed virus can be calculated based on the amount of virus in suspension when the system is at equilibrium, meaning how much virus is in suspension when the rate of adsorption and desorption are equal (Gerba 1984). Electrostatic forces, such as repulsive double layer interactions that form between the viral particle and the surface as well as attractive Van der Waals forces, also play a significant role in virus adsorption. When a virus is immersed in a solution, it will attract oppositely charged ions to its surface in a compact layer called the "Stern Layer" (**Figure 1.5**). In order for the system to remain neutral another layer containing counterions, the "Gouy Layer", forms extending from the virus into the bulk solution. The extent to which the "Gouy Layer" extends into the bulk solution determines the distance and force with which the virus and surface repel each other. When the bulk solution of counterions is increased, the "Gouy Layer" decreases because it needs fewer counterions to neutralize the charge of the virus colloid. As demonstrated in **Figure 1.5** as the distance between the virus and the surface decreases and the attractive Van der Walls forces are allowed to take place (Gerba 1984).

Figure 1.5 Forces affecting the virus-binding surface interaction

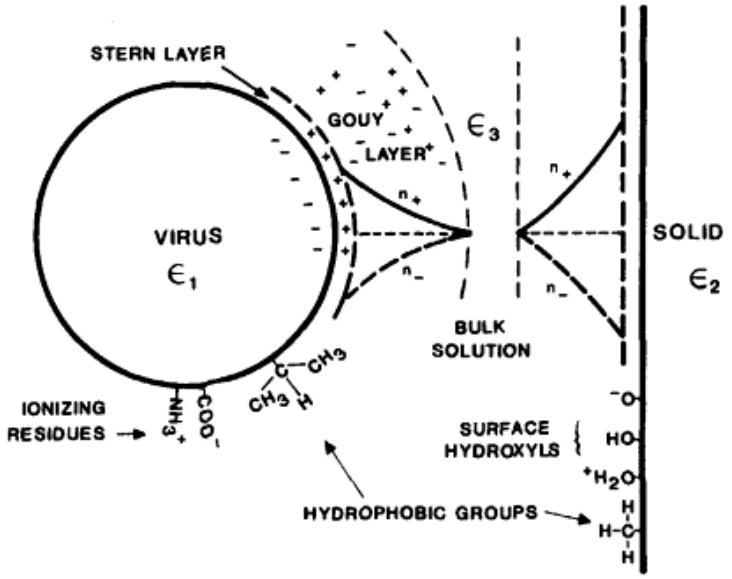


FIG. 1. Diagrammatic representation of double layers and hydrophobic groups about a virus and solid. The presentation shows ionizing residues and surface hydroxyl groups thought responsible for charge development as explained by the electrical double-layer theory of colloidal chemistry; the Stern layer of bound cations and anions, and the Gouy layer with cation (n_+) excesses and anion (n_-) deficits. ϵ_1, ϵ_2, and ϵ_3 are the complex dielectric susceptibility functions important in controlling the differences in magnitudes of van der Waals interactions with different materials. Hydrophobic groups on virus and solid may interact by exclusion of water molecules.

(Gerba1984)

In addition to electrostatic interactions, hydrophobic bonding has also been indicated as a significant interaction influencing viral binding. Studies have found that the use of chaotrophic salts promotes the elution of virus from membrane filters (Gerba, 1984). This effect is attributed to the ability of chaotrophic salts to disrupt the structure of water thereby enhancing the accommodation of hydrophobic groups. Antichaotrophic salts conversely will promote virus adsorption by promoting the sequestering of hydrophobic groups.

Shields and Farrah (1983) demonstrated that the importance of the electrostatic and hydrophobic interactions vary depending on pH. They assessed the effect of salts and detergents on the elution of Poliovirus (PV) from nitrocellulose filters. They found that at

pH 4, the addition of a 1.0 M NaCl or 0.1% Tween® 80alone was not enough to allow for sufficient elution of membrane bound PV resulting in a 2 and 13% elution rate, respectively. When the two solutions were used together, however, 100% of PV was able to eluted from the filter. Additionally, they found that even at a low concentration of Tween® 80 (0.005%) when combined with 1.0 M NaCl, PV could still be eluted by 91%. At pH between 9-11 they observed that 0.1% Tween® 80alone was more effective than at pH 4 allowing for 66% of the virus to be recovered. When using 1.0 M NaCl alone at pH 9, however, they were only able to elute 35% of PV. At low pH (pH 4) it appears that both electrostatic and hydrophobic interaction are significant as targeting hydrophobic bonds alone was not effective at eluting virus from membrane filters. In order to promote efficient elution, hydrophobic interactions must first be disrupted followed by the addition of a charged species or a change in pH to disrupt the electrostatic interactions. At high pH (9-11), however, hydrophobic interactions seem to dominate, as the addition of Tween® 80 alone allowed for efficient elution (Gerba, C. 1984).

Inactivation Methods

Physical Inactivation Methods

The persistence of HuNoV in the environment can be attributed partially to its ability to resist many standard inactivation methods, such as acidification, heating, and cooling. Mormann et al. (2010) evaluated the effect of physiochemical inactivation of GII HuNoV in artificially contaminated foods. The result of this study found that typical preservation methods, such as refrigeration and cooling, were ineffective at significantly reducing HuNoV levels. Additionally low pH was found ineffective, which is to be

expected as HuNoV survives the environment of the stomach. Different heating methods were also evaluated for effectiveness. Heating methods such as pasteurization (74 °C for 1 min) achieved a less than 1 log reduction whereas boiling (100 °C for 30 min) and baking (200 °C for 30 min) inactivated the virus completely (Mormann et al. 2010). This study demonstrated that HuNoV is resistant to typical food processing methods and as such poses a major threat as a cause of food-borne disease.

High Pressure Processing (HPP) is a non-thermal method of producing food that is free of pathogens. HPP has been used to inactivate enteric viruses such as adenovirus, poliovirus and rotovirus in foods. The mode of inactivation for this method is presumed to be the denaturation of the viral coat proteins induced by high pressure. The effectiveness of HPP is influenced by the temperature, pH, and composition of the food matrix (Hirneisen et al. 2010). HuNoV surrogates FCV and MNV-1 have both been shown to be inactivated by HPP. FCV is completely inactivated (7 logs reduction) at 275 MPa for 5 minutes while MNV-1 has displayed over 5 logs reduction at 450 MPa for 15 min (Kingsley et al. 2002; Sanchez et al. 2011). Studies using HuNoV have found slightly varying results. Sanchez et al. (2011) found that while 5 logs inactivation of MNV-1 was achieved at 400 MPa, HuNoV displayed <0.5 log reduction for pressures up to 450 MPa (Sanchez et al. 2011). Leon et al. (2011), however, demonstrated that HPP was successful in inactivating HuNoV in oysters. Oysters seeded with approximately 10^8 RT-PCR units/ml of HuNoV were subjected to HPP and fed to volunteers. It was found that pressures of 600 MPa but not 400 MPa were successful in inactivating HuNoV and preventing illness (Leon et al. 2011).

The use of nonionizing radiation (UV) or ionizing radiation (gamma) is another physical method that has been employed in the food industry. Inactivation of HuNoV by both types of irradiation occurs by the generation of free radicals that damages the nucleic acid. However, some studies suggest that inactivation of enteric viruses by UV may occur primarily through damage to the viral capsid which allows the RNA to be more susceptible to damage. (Hirneisen et al. 2010; Pecson et al. 2009; de Roda Husman et al. 2004). An important difference in these two methods is that UV light only inactivates pathogens on the surface. Gamma irradiation has the ability to penetrate into the internal structure of foods, which is important as internalization of pathogens has been proposed as a possible route of fresh produce contamination. Feng et al. (2011) demonstrated that MNV-1 achieved a maximum reduction of 1.7-2.4 log in viral titer when irradiation (5.6 kGy) was applied to artificially contaminated lettuce, spinach, and strawberries. Using 11.2 kGy, MNV-1 achieved between 3.6-4.1 log reduction in viral titer on produce samples. When the amount of irradiation applied was increased to 22.4 kGy, complete inactivation of MNV-1 was achieved on lettuce and strawberries, however, 2.4 log pfu was still detectable on spinach. These results demonstrate that irradiation does have a detrimental effect on viral particles but it does not achieve reduction required for complete inactivation within the FDA approved dose for use on fresh produce (4 kGy). Lee et al. (2008) evaluated the inactivation of MVN in suspension subjected to irradiation by 254-nm UV light. They found approximately a 3.3 log reduction in viral titer of MNV suspended in PBS could be achieved using 25 mJ/cm^2 UV. De Roda Husman et al. (2004) also evaluated the inactivation of MS2, FCV, and Canine Calicivirus (CaCV) using 253.7 nm UV and gamma irradiation in tap water as

well as low and high protein content solutions. They observed that using UV irradiation a 3 log reduction in viral titer of MS2, FCV, and CaCV was achieved at a fluence of 65, 12, and 20 mJ/cm^2, respectively, regardless of the suspending media. When using gamma irradiation they found that a 3 log reduction in viral titer of MS2 was achieved using a dose of 120 Gy in tap water and low protein content suspension. In order to achieve a 3 log reduction in viral titer of FCV and MNV under the same conditions, a dose of 500 and 300 Gy, receptively, was required. When the viruses were contained in high protein content solution they observed no reduction in viral titer of MS2 and ≤2 log reduction in viral titer of FCV and CaCV at a dose of up to 1 kGy. The authors noted that the inability of the viral inactivation by gamma irradiation in high protein stocks resulted from the interaction of the OH free radicals with particles in the solute such as proteins, sugars, and fats.

HuNoV is a small RNA virus with a highly stable capsid (Feng et al. 2011). As RNA is less susceptible to degradation by free radicals, these qualities may make HuNoV less susceptible to inactivation methods through irradiation (Hirneisen et al. 2010).

Chemical Inactivation

In order for a disinfectant to be considered effective it must achieve at least 4 log reduction in viral titer (EPA. 1981; Poschetto et al. 2007). The efficacy of a disinfectant varies greatly depending on the virus, surface, concentration, contact time, and suspending medium. HuNoV has displayed resistance to several commonly used disinfectants. Due to the fact that multiple surrogates in addition to HuNoV are used in laboratory studies on different surface types, it can often be hard to draw a concise

conclusion. In general, limited reduction (1 log) of HuNoV has been observed using quaternary ammonia compounds, ethoxylated alcohols, and anionic detergents (Barker et al. 2004; Chessbrough et al. 2000; Girard et al. 2010). All of these disinfectants are lipophilic products targeting the envelope of the virus, which HuNoV is lacking.

Quaternary ammonium compounds are generally ineffective at achieving significant reduction (>1 log) in FCV, MNV or HuNoV as shown in **Table 1.3**. Increased efficacy against FCV and MNV can be achieved, between 2-3 log reduction, using increased concentrations and contact times. Some studies reported complete inactivation of FCV (>5 log reduction) from chemical inactivation, however, these inactivation rates are usually attributed to synergistic effects of pH or other biocides.

Table 1.3 QAC disinfection of HuNoV and surrogates

Surface	Virus	Treatment	Time	Significant results	Reference
Stainless steel	MNV NV	0.02 and 0.08% N-alkyl dimethyl benzyl ammonium chloride	5 and 10 min	1 log reduction of MNV after 10 min and <0.05 after 5 min No reduction for NV	Girard, M. et al. (2010)
Stainless steel Strawberry Lettuce	FCV	450, 900, and 800 ppm 9% QAC and 400, 800, and 1,600 ppm 10% QAC QAC: n-quaternary ammonium compound alkyl dimethyl benzyl ammonium chloride	10 min	>/ 2 log reduction of FCV at 1,800 and 1,600 ppm 9% and 10% QAC, respectively </ 1 log reduction for all other concentrations	Gulati, B. et al. (2001)
Petri dish with 5% soiling	FCV	850 ppm QAC R-82	10 min	>/ 6.4 log reduction of FCV	Jimenez and Chiang (2006)
Glass coverslips	FCV	848, 4,240, and 8,480 ppm Sentramax	10 min	1.9 log reduction of FCV with 848 ppm Sentramax Higher concentrations were less effective	Solomon, E. et al. (2008)
Polystyrene petri dish	FCV	3,000 and 1,000 ppm 80 and 50 % QAC in liquid. 1,000 ppm 80 and 50% QAC delivered by trigger	1 min	Liquid: 1.17 and <2.27 log reduction of FCV using 80 and 50% QAC	Whitehead and McCue (2010)

		and aerosol respectively		respectively Trigger and aerosol: >3.0 log reduction of FCV, however, it should be noted that their pH was significantly higher (11.0 and 12.0)	
Suspension test	MNV	Asphene 381 (Quat, alkylamin, non-ionic detergent)	0.5,1, and 3, 15, 30, 60 min	1 log reduction at 1 min only. All other times achieved <1 log reduction	Belliot, G. et al. (2008)
Suspension test	FCV	Pinocleen	1 min	No inactivation	Doultree, J. et al. (1999)
Suspension test	FCV MNV HuNovGII.2 HuNovGII.4	0.1X, 1X, 10X concentrations of Bardac 208M 20% alkyl [C14 50%, C12 40%, C16 10%] dimethyl benzyl ammonium chloride, 15% octyl decyl dimethyl ammonium chloride, 6% dioctyl dimethyl ammonium chloride, 9% didecyl dimethyl ammonium chloride	30 s	<0.5 log reduction of all viruses at all concentrations	Tung, G. et al. (2013)

The efficacy of ethanol and isopropanol against FCV, MNV, and HuNoV ranged from <0.5 to >4 log inactivation, as shown in **Table 1.4**. Alcohols are a common active ingredient in hand sanitizers and are also used as hard-surface disinfectants. Ethanol and isopropanol are the two most commonly used alcohols usually with concentrations ranging from 60-99.5%. While there have been many laboratory studies determining the efficacy of alcohols on HuNoV and its surrogates, the results vary greatly. Belliot et al. (2008) demonstrated that MNV in suspension could be inactivated by >3 and >4 log after 0.5 min using 60% ethanol and isopropanol, respectively. Gehrke et al. (2004) supported these findings reporting that concentrations of 50-80% ethanol, 1-propanol, and 2-propanol could inactivate FCV in suspension at contact times of 0.5-5 min. In addition they reported that 70% ethanol and 1-propanol could inactivate FCV on fingertips by >3 log reduction after only 30 sec of contact time. Magulski et al. (2009) showed that MNV dried onto a stainless steel surface under both clean and dirty conditions achieved a >4 log reduction using 50 and 30% ethanol and 1-propanol, respectively. Lower efficacies, however, have been demonstrated in multiple other studies. Park et al. (2010) investigated the inactivation of FCV, MNV, HuNoV GII.2 and GII.4 in suspension and found that concentrations of 50-90% of ethanol and isopropanol achieved <3 log reduction for FCV and MNV and <2 log reduction for HuNoV after 5 min of contact time. Lages et al. (2008) reported similar results on fingertips, finding a maximum of 1.3 log reduction using 99.5% ethanol. On stainless steel, MNV and HuNoV showed no significant reduction when treated with an ethoxylated alcohol after 10 min.

Although the results of these studies vary greatly, it can be concluded that HuNoV is not completely inactivated (>3 log) by alcohols. Complete inactivation of HuNoV

using alcohols can be achieved using formulations containing additional antimicrobial compounds, most likely due to synergistic effects. This was demonstrated by Liu et al. (2011), who reported >3 log reduction of HuNoV GI and GII.4 using a formulation of 70% ethanol plus additional chemicals.

Table 1.4 Ethanol disinfection of HuNoV and surrogates

Surface	Virus	Treatment	Contact time	Significant Results	Reference
Suspension test	MNV	60, 30, and 10% ethanol and isopropanol	0.5-3 min	Ethanol: 60 % achieved >4 log inactivation of MNV for all contact times 30% achieved <1 log inactivation of MNV for all contact times 10%: no inactivation at all contact times Isopropanol: 60% achieved > 3 log inactivation of MNV after 0.5 min and >4 log inactivation of MNV after 1 and 3 min 30 % and 10 % achieved < 1 log inactivation of MNV for all contact times	Belliot, G. et al. (2008)
Suspension test	FCV	75% ethanol	1 min	1.25 log reduction in virus titer	Doultree, J. et al. (1999)
Suspension test	FCV	70% ethanol	0.5-60 min	2 log reduction after 8 min 3 log reduction after 30 min	Duzier, E. et al. (2004)
Suspension test and fingertips	FCV	50, 70, and 80% ethanol, 1-propanol and 2-propanol	0.5, 1.0,3.0, and 5.0 min in suspension 30 sec for fingertips	Ethanol: 4 log reduction achieved with 50% and 70% after 3 min and 80% after 5 min 1-Propanol: 4 log reduction achieved with 50 and 70% after .5 min and 80% after 3.0 min 2-propanol: 4 log reduction achieved with 50% after 3.0 min, 70% at	Gehrke, C. et al. (2004

				5.0 min and 80% >5 min Fingertips: 70% ethanol and 1-propanol achieved >3 log reduction in 30 s	
Stainless steel	MNV HuNoV	Ethoxylated alcohols	5 and 10 min	No significant reduction	Girard, M et al. (2010)
Fingertips	FCV	99.5 and 62% Ethanol 91 and 70% Isopropanol	0.5 min and 2 min	99.5% ethanol achieved a 1 log reduction after 0.5 min and 1.3 log after 2 min. All other alcohols achieved <1 log reduction	Lages, et al. (2008)
Suspension test and Finger tips	HuNoV	3, 17, 31, 47, 62, and 95% ethanol (suspension test) 60% ethanol (fingertips)	0.5 min	<0.5 log reduction	Liu, P. et al. (2010)
Fingertips	Norwalk virus (NV) SMV (HuNoV .GII.2)) HuNoV. GII.4	62, 63, 70(a), 70 (b) 85, and 95% ethanol (a and b are formulations containing ethanol and other biocides)	15 sec	70%(b) achieved >3 log reduction of NV 70%(b) achieved >2 log reduction of SMV (HuNoV.GII.2) 70%(b) achieved >4log reduction of HuNoV.GII.4	Liu, P et al. (2011)
Suspension test	MNV	75% ethanol	30 sec	0.91 log reduction	Macinga, D et al. 2008

Stainless steel clean (0.03%BSA) and dirty (0.03%BSA +0.3% erythrocytes	MNV	40-60% ethanol 10-60% 1-propanol 20-60% 2-propanol	5 min	50% ethanol and 30% 1-propanol achieved >/ 4 log reduction under clean and dirty conditions	Magulski, T. et al. (2009)
Suspension test	FCV MNV HuNoV. GII.4	50, 70, an 90% ethanol and isopropanol	5 min	FCV: 50% and 70% isopropanol achieved >/2.2 log reduction MNV: >/70% ethanol achieved >/2.6 log reduction HuNoV.GII.4: 90% ethanol and isopropanol achieved a 1.2 and 1.8 log reduction respectively	Park, GW et al. (2010)
Suspension test	FCV MNV HuNoV. GII.2 HuNoV. GII.4	50, 70, 90% ethanol	30 sec	FCV, HuNoV.GII.2 and GII.4 achieved <0.5 log inactivation MNV achieved approximately 2 log reduction with 70 and 90% ethanol	Tung, G. et al. (2013)
Polystyrene petri dish	FCV	Formulation	1 min	>3 log reduction	Whithead and McCue (2010)

Chlorine Inactivation

Chlorine bleach is a widely used disinfectant across many industries. The active ingredient, sodium hypochlorite, is a strong oxidizing agent with a wide range of efficacies depending on concentration, contact time, and soiling. The mode of action associated with chlorine is somewhat debatable. A recent study published by Ursula Jakob at University of Michigan suggests that chlorine causes bacterial proteins to lose their structure and form large aggregates resulting in cell death (University of Michigan. 2008). However, the mechanism may not be the same for viruses. Research suggest that inactivation of viruses by chlorine is associated with both the capsid and the RNA. O'Brien and Newman (1979) demonstrated that poliovirus and picornavirus treated with chlorine had similar sedimentation coefficients to that of an empty capsid, indicating that inactivation may be due to the release of RNA, however, upon further investigation they found that a decrease in RNA did not correspond with a loss in infectivity. Instead it was determined that inactivation was due to cleavage of RNA that was still associated to the capsid. Results reported by Li et al. (2002) on the detection of hepatitis A virus (HAV) suggested that the mechanism may be different depending on the virus. Their study found that after chlorine treatment, ELISA could still detect HAV antigens even after the loss of infectivity. This indicated that damage to nucleic acid was the primary method of inactivation rather than damage to the capsid proteins.

The use of chlorine bleach has been shown to successfully eliminate HuNoV across several studies; however, the concentrations needed and contact times vary. The studies summarized in **Table 1.5** generally agreed that 1,000-5,000 ppm of chlorine bleach is sufficient to inactivate HuNoV on hard non-porous surfaces (Barker et al. 2004;

Hirneisen et al. 2010). The concentration needed can vary based on the surface or in what medium the viral particles are contained. Girard et al. (2010) demonstrated that complete inactivation (3 log reduction) of HuNoV attached to stainless steel could be achieved by using 3% bleach (30,000 ppm) for 10 minutes. Shorter contact times were sufficient to reduce HuNoV levels but not to completely inactivate the virus. The concentration used in the above experiment was much higher than current recommendations for cleaning environmental contamination, and it is unclear why such high concentrations were used. Barker et al. (2004) showed that 5,000 ppm of bleach was insufficient at eliminating HuNoV suspended in fecal matter. Only by removing the initial soiling and cleaning with a detergent and then applying 5,000 ppm bleach HuNoV could be inactivated. In order to achieve complete inactivation a mixture of 5,000 ppm bleach plus 0.04 % anionic detergent mixture was needed. These results were supported by a study by Poschetto et al. (2007) where high concentrations of bleach (>5500 ppm) were unable to decrease the titer of HuNoV in fecal suspension, after the concentration of fecal matter was reduced, HuNoV reduction of up to 4 logs was achieved (Barker et al. 2004; Poschetto et a. 2007). Chlorine bleach has also been used in small amounts to decontaminate water. Kitajima et al. (2010) demonstrated that 0.5 ppm of chlorine was able to achieve >3 log reduction of HuNoV after 30 minutes. Clinical trials have indicated that higher levels would be needed to inactivate HuNoV in artificially inoculated water. When individuals were given HuNoV inoculated water, they still became ill when chlorine from 3.75-6.25 ppm was used. Only upon increasing chlorine concentrations from 6.25 ppm to 10 ppm of chlorine did no volunteers develop illness (Hirneisen et al. 2010). It is clear that many

different factors can affect the inactivation of HuNoV and that standardization is needed for the evaluating the use of bleach or other disinfectants.

Table 1.5 Chlorine disinfection of HuNoV and surrogates

Surface	Virus	Treatment	Time	Significant results	Reference
Melamine surface with fecal soiling	HuNoV .GII	HDC (hypochlorite/disinfectant cleaner containing 5,000 ppm available chlorine and 4% anionic detergent)	1-5 min	HuNoV still detected in 28% of samples using HDC alone. Pre cleaning with detergent allowed complete inactivation	Barker, J, et al. (2004)
Suspension test	MNV	2,600 ppm active chlorine	0.5,1, and 3 min	>4 log inactivation	Belliot, G. et al. (2008)
Suspension test	FCV	2 hypochlorite compounds ranging from 100, 250, 50, 1000, 5000 ppm	1 min	5,000 ppm achieved 5 log reduction. 1,000 ppm achieved 5 log reduction for freshly reconstituted granular hypochlorite but not pre-reconstituted solution. </ 500 ppm achieved <3 log reduction	Doultree, J. et al. (1999)
Suspension test	FCV HuNoV .GII.4	0-300, 3,000 and 6,000 ppm chlorine	10 and 30 min	<2 log inactivation of FCV achieved at 300 ppm >5 log reduction using 3,000 ppm for FCV HuNoV RNA undetectable by RT PCR after 6,000 ppm	Duizer, E. et al. (2004)
Lettuce	FCV MNV	15 ppm active chlorine	2 min	FCV achieved 2.9 log inactivation MNV achieved 1.4 log inactivation	Fraisse, A. et al. (2011)
Stainless steel	MNV HuNoV	30,000 ppm chlorine	5 and 10 min	FCV completely inactivated at 5 and 10 min HuNoV complete inactivation (>3 log) after 10 but not 5 min (<2 log)	Girard, M. et al. (2010)

Stainless steel Strawberry Lettuce	FCV	200, 400, 800, and 5,000 ppm free chlorine	10 min	Only 800 ppm achieved >1 log reduction on produce and stainless steel 5,000 ppm achieved 3.4 log reduction on stainless steel	Gulati, B. et al. (2001)
Petri dish with 5% soiling	FCV	100 and 1,000 ppm hypochlorite	10 min	3.2 and 6.6 log reduction for 100 and 1,000 ppm, respectively	Jimenez and Chiang (2006)
Drinking water	MNV HuNoV	0.1 or 0.5 ppm chlorine	0.5, 1, 2, 5, 10, 30, 60, 120 min	MNV achieved >4 log reduction for 0.1 ppm after 120 min and 0.5 ppm at all contact times HuNoV achieved >3 log reduction with 0.5 ppm after 30 min	Kitajima, M. et al. (2010)
Suspension test	MNV HuNoV	Chlorine and chlorine dioxide	1-2.5 min at 5 and 20 C	Chlorine at 0.184-0.193 and chlorine dioxide at 0.255-0.288 ppm achieved >3 log inactivation. Longer contact time was needed at 5 ° C vs. 20 ° C 0.191 ppm achieved inactivation of HuNoV below LOD	Lim, M. et al. (2010)
Suspension test	HuNoV	3, 22, 51, 160, 1,600 ppm chlorine	30 sec	5 log reduction for 160 and 1600 ppm	Liu, P. et al. (2010)
Stainless steel, ceramic tiles, and suspension test	MNV MS2 HuNov. GII.4	20-200 ppm hypochlorous acid solution and fog	10 min	20-200 ppm HAS achieved >/ 3 log reduction in MS2 and HuNoV.GII.4 in suspension after 20 sec. Surface test need 1-10 min depending on concentration. Fogging achieved >4.5 log reduction for MNV, MS2, and HuNoV.GII.4	
Stainless steel with 10%	FCV MNV	250, 500, 1000, 2,500, 5,000 ppm	2, 4, and 10	5,000 ppm inactivated FCV, MNV, MS2 by 3 log after 1.9, 3.2, and 4.5 min,	Park and Sobsey

soiling	MS2 HuNoV GII.4	free chlorine	min	respectively, and HuNoV.GII.4 by 1.4 log after 4 min	(2011)
Suspension test with or without 40% FBS, with 25% feces	FCV NV	12,000 ppm sodium hypochlorite	15,30, 60, and 120 min	FCV achieved >/ 4 log inactivation with 4,500-5,000 ppm after 15 min without feces and a 4 log reduction using 5,500 ppm with 25% feces FCV and HuNoV achieved >/ 3 log reduction after 6,000 ppm for 15 min with 10% feces	Poschetto, L. et al. (2007)
Suspension test	FCV MNV HuNov. GII.2 HuNov. GII.4	5, 75, 250, 500, 1,000 ppm sodium hypochlorite	30 s	500 ppm and 250 ppm achieved >3 log inactivation of FCV and MNV, respectively >/500 ppm achieved 3 log reduction of HuNoV.GII.2 and GII.4	Tung, G. et al. (2013)
Polystyrene petri dish	FCV	100 and 1,000 ppm	1 min	100 and 1,000 ppm achieved >2 log and >4 log reduction, respectively	Whitehead and McCue (2010)

Additional Chemical Inactivation

Although the previous section focused on the resistance of HuNoV and its surrogates to chemical inactivation, there are several laboratory studies that have demonstrated the effectiveness of different chemical compounds at inactivating the virus. Some widely used chemical methods that have shown significant efficacy include iodine, glutaraldehyde, peroxygens, and chlorine dioxide. Efficacy of these products as with others is dependent on the viral load, suspending medium, concentration, and contact time. A detailed account of studies is shown in **Table 1.6**.

Iodine-based products have been shown to completely inactivate FCV and MNV on hard surfaces using iodine concentrations from 0.8-1%. Gulati et al. (2001), however, demonstrated that high concentrations might be needed as FCV was shown to achieve only a 2 log reduction on food-contact surfaces using 300 ppm of iodine+phosphoric acid. Glutaraldehyde (GDA) is a component in many commercially available sanitizers and has displayed various efficacies against HuNoV and its surrogates. The majority of studies using GDA or GDA-based sanitizers agree that FCV and MNV can be completely inactivated using 0.1-2% GDA. Poschetto et al. (2007) demonstrated that 0.1 % could achieve >3 log after 15 min even in presence of soiling (40% FBS and 25% feces). However, when evaluated against HuNoV in 25% fecal suspension, 2% GDA achieved only a 2 log reduction after 1 hr.

Peroxyacetic acids rely on either peracetic acid (PAA) alone or sometimes in combination with hydrogen peroxide. Fraisse et al. (2011) found that FCV and MNV inoculated onto lettuce achieved a reduction of 3.2 and 2.3 logs, respectively, when washed with 100 ppm PAA. Studies on food-contact surfaces with FCV have mirrored

these results showing complete inactivation (>3 log reduction) with concentrations of PAA+H_20_2 from 0.03-0.1 %. Magulski et al. (2009) indicates that higher concentrations of PAA+ H_20_2 can inactivate MNV and achieved a ≥ 4 log reduction in 5 minutes. As PAA+ H_20_2 compounds rely on oxidative activity, their efficacy can be affected by soiling. Poshcetto et al. (2007) also evaluated the efficacy of "Oxystrong FG" (14-16% PAA+22-24% H_2O_2+<15% acetic acid) against FCV and HuNoV under clean and dirty conditions. "Oxystrong FG" at 0.1% concentration was able to achieve >3 log reduction in FCV titers after 15 min under clean conditions and in the presence of artificial soiling (40% FBS). In the presence of fecal soiling (25%) a 1% solution for 60 min contact time achieved the same reduction in FCV and HuNoV.

Chlorine dioxide (ClO_2) is another virucide, which has been evaluated for use against HuNoV and its surrogates. ClO_2 most often associated with its use for disinfecting water, however, it can be used to disinfect surfaces as a liquid or gas. The use of ClO_2 in water may be more suitable than traditional chlorine as it doesn't react with ammonia nor does it form halogen byproducts (Lim et al. 2010; Thurston-Enriquez et al. 2005). Thurston-Enriquez et al. (2005) evaluated the use of ClO_2 to inactivate FCV in suspension. They found that 0.72 ppm ClO_2 exhibited a maximum efficacy at pH 8 and 15 **°C** achieving a >4.15 log reduction in viral titer after 15 sec. The efficacy of ClO_2 was improved at pH 8 and a temperature of 15 **°C** as opposed to pH 6 and 5 **°C**. The use of ClO_2 gas as a surface disinfectant was investigated by Morino et al. (2009). FCV was inoculated onto glass dishes and subjected to inactivation by ClO_2 gas under a variety of conditions. In the wet state and conditions of moderate relative humidity (45-55%) (MRH), FCV containing 0, 0.5, and 1% FBS could be inactivated by 5.7, 3.6, and 2.2

logs using 0.5, 2.8, and 4.2 ppm ClO_2 after 6h, respectively. Inactivation of FCV in the dry state was proven to be much more difficult. When FCV was dried onto glass at MRH, the maximum reduction in viral titer achieved was 1.3 (w/o FBS) and 2.1 log (w/FBS) using 8 ppm ClO_2 after 24 h. When the same conditions were assessed at 75-85% RH, FCV was inactivated by 4.6 (w/o FBS) and 6.0 log (w/5%FBS). MNV and MS2 have also been shown to be susceptible to ClO_2 in suspension. Lim et al. (2010) found that MNV and MS2 could be completely inactivated in suspension using ClO_2, and that the rate of inactivation was time/temperature dependent. Complete inactivation of MNV by 3.5 log was achieved at 5 and 20 **°C** after 1 and 0.5 min using 0.288 and 0.255 ppm ClO_2, respectively. MS2 demonstrated a 3.5 and 4.7 log inactivation after 2 min using 0.174 and 0.178 ppm ClO_2, respectively. The authors also noted the concentration of ClO_2 used in this experiment was lower than what is required by the EPA for drinking water suggesting it is a successful method for decontamination. The inactivation of HuNoV GII.4 by ClO_2 was evaluated by Nowak et al. (2011). In their study they used a combination of RT-qPCR and RNase treatment in order to determine if virolysis through destruction of the capsid was achieved by several common methods of inactivation. They found that treatment of HuNoV GII.4 with 200 ppm ClO_2 resulted in incomplete virolysis. After treatment 35.63% of viral RNA was recovered with no additional reduction after RNase treatment. This demonstrated that the detectable RNA was from an intact virus particle indicating that it is likely still infectious. The results from the above mentioned studies indicate that ClO_2 may be an effective method for inactivating HuNoV, however, the efficacy will be influenced by many of the same factors that influence

chlorine, such as temperature, pH, and especially organic content. Additionally when using ClO_2 gas the RH will also be an important factor.

Table 1.6 Additional chemical disinfection of HuNoV and surrogates

Surface	Virus	Treatment	Time	Significant results	Reference
Lettuce	FCV MNV	100 ppm Peroxyacetic based disinfectant 10.9% Acetic Acid 5% Peracetic acid 20.7% H_2O_2	2 min	3.2 log inactivation of FCV 2.3 log inactivation of MNV	Fraisse, A. et al. (2011)
Suspension test	MNV	1X Betadine (1.0 %povidone iodine)	0.5,1, and 3 min	>4 log reduction of MNV at all contact times	Belliot, G. et al. (2008)
Suspension test	FCV	"Aidal" (0.5% GDA) "Sanichick" (0.8% iodine)	1 min	Both products achieved a 5 log reduction	Doultree, J. et al. (1999)
Formica coupons at high (10^7) and low (10^5) titer	FCV MNV MS2	1,2, and 5% trisodium phosphate 2% GDA	0.5 and 1 min	5% TSP achieved approximately 6 and 5 log reduction for FCV, MNV, and MS2 on high and low titer respectively FCV and MS2 were inactivated at >/2 % TSP, however, MNV needs 5% for inactivation 2% GDA achieved approximately 6 log reduction for FCV and MNV but a max 3.74 log reduction for MS2	D'Souza and Su (2010)
Stainless steel Strawberry Lettuce	FCV	1X, 2X, and 4X 15% peroxyacetic acid+11% H_2O_2 (PAHP)	10 min	3 log reduction of FCV using 4X PAHP and 2 log reduction using 300 ppm IPH	Gulati, B. et al. (2001)

		75, 150, and 300 ppm 1.75% iodine+6.5% phosphoric acid (IPH)			
Stainless steel under Clean conditions (0.03%BSA)	MNV	50, 200, 500, 1,000, and 1,500 ppm peracetic acid (PAA) 125, 500, 1,000, 2,000, and 2,500 ppm GDA	5 min	>/ 4 log inactivation of MNV with 1,000 and 2,500 ppm PAA and GDA, respectively	Magulski, T. et al. (2009)
Suspension test with or without 40% FBS, with 25% feces	FCV NV	3, 4, and 5% "Venno Vet 1 Super" 55-60% formic acid+7% glyoxylic acid 0.1, 0.5, 1, and 2% "Veno FF Super" 20-25% GDA+12% oligomers 1 and 2% "Oxystrong FG" 14-16% PAA+22-24% H_2O_2+<15% acetic acid	15,30,60, and 120 min	Without organic matter: 0.5% VV1S, 0.1% VFFS, and 0.1% OFG achieved >/ 3 log reduction in FCV after 15 min In the presence of organic matter 4% VV1S and 1% OFG were needed NV in presence of 25% feces achieved a >/ 3 log reduction with 5% VV1S, 2% VFFs and 1% OFG after 60 min contact time	Poschetto, L. et al. (2007)
Glass coverslips	FCV MS2	0.1, 0.25, 0.5 and 1% "Vikron" (stabilized blend of peroxygen compounds, surfactant, organic acids, and inorganic buffer)	10 min	>4 log and >5 log reduction of FCV and MS2, respectively using 1% Vikron	Solomon, E. et al. (2008)

Diluted stool sample	HuNoV GII.4	200 ppm liquid ClO_2	5 min at 20 **°C**	35.63% of initial titer of HuNoV GII.4 recovered after treatment	Nowak et al. (2011)
Glass	FCV	0.26, 0.5, 2.8, 4.8, 8 ppm gaseous ClO2	6 and 24 h 20 **°C** 45-55% and 75-85% RH 0, 0.1, 0.5, 5% FBS wet and dry inoculum	Wet and 45-55% RH: FCV w/ 0,0.5, and 1% FBS was inactivated by 5.7, 3.6, and 2.2 log using 0.5, 2.8, and 4.2 ppm ClO_2 after 6 h Dry: At 45-55% RH FCV w/ and w/o 5% FBS was inactivated by 2.1 and 1.3 log using 8 ppm ClO_2 after 24 h At 75-85% RH FCV w/ and w/o 5% FBS was inactivated by 6.3 and 6.5 log using 0.26 ClO_2 after 24 h	Morino et al. (2009)
Suspension		0.72-1.01 ppm liquid ClO_2	15 s 5 and 15 **°C** pH 6 and 8	FCV was inactivated by >4.15 log using 0.72 ppm ClO_2 at 15 **°C** and pH 8	Thurston-Enriquez et al. (2005)

Recovery methods

Environmental Sampling

Recovery of HuNoV from surfaces in both laboratory and environmental studies has traditionally been accomplished by swabbing. Swabbing has been shown to successfully recover HuNoV on multiple surface types including hard and soft surfaces, fingertips, and food surfaces. Recovery of viruses by swabbing can be influenced by the type of swab used, the surface being recovered from, and the eluent used. Julian et al. (2011) conducted a literature review of surface sampling methods used to recover viruses from fomites and used a subset of those methods to recover MS2 as a model virus. Results from this study showed the type of swab used was the most significant factor affecting recovery rates. Polyester swabs yielded a significantly higher fraction of positive samples (28%) as opposed to either cotton or rayon-tipped swabs (18 and 6% respectively). Fowler (2012) demonstrated that surface area of the swab also plays a role in recovery of HuNoV. They found that a foam-tipped swab, which had a greater surface areas, recovered significantly more HuNoV from stainless steel, smooth ceramic, and rough plastic than a nasopharyngeal swab.

While Julian et al. (2011) determined that sampling method had the most significant influence on recovery; additional studies have highlighted the role of surface type as well as eluent type on the efficiency of recovery. Swabbing has been shown to be more effective when using a wet versus a dry swab (Fowler. 2012). Swabs are typically moistened using phosphate buffered saline (PBS), however, many investigators modify the eluent components based on the pathogens being recovered. In the study by Julian et al. (2011) eluent type was determined to be not significant, however, it was noted that

higher recovery rates were seen using Ringers solution or 0.85% saline versus other types of media or water. Taku et al. (2002) also evaluated the role of eluents used in recovery. They found that recovery using 0.05 M glycine at pH 6.5 resulted in a higher recovery (42%) of FCV versus the same buffer at pH 8.5 (28%) or cell culture media (10%). When recovering FCV from fresh produce, Fino and Kniel (2008) observed that using media containing 2% fetal bovine serum (FBS) was better than PBS or beef extract (0.05M glycine). Eluents are typically adjusted for pH, salt content, and presence of amino acids as these are all factors capable of influencing virus binding (Gerba, C. 1984).

Detection Methods

Traditional Methods

There are several standard methods used for the detection of HuNoV, each with advantages and disadvantages. Many studies recommend that at least two methods of detection be used if there are questionable results (Rabenau et al. 2003).The first method used for identifying HuNoV is transmission electron microscopy (TEM). This method is advantageous because it is relatively rapid and allows the particle to be visualized. TEM, however, is not usually practical for detection purposes because it is somewhat costly, requires a trained operator, and only one sample can be analyzed at a time.

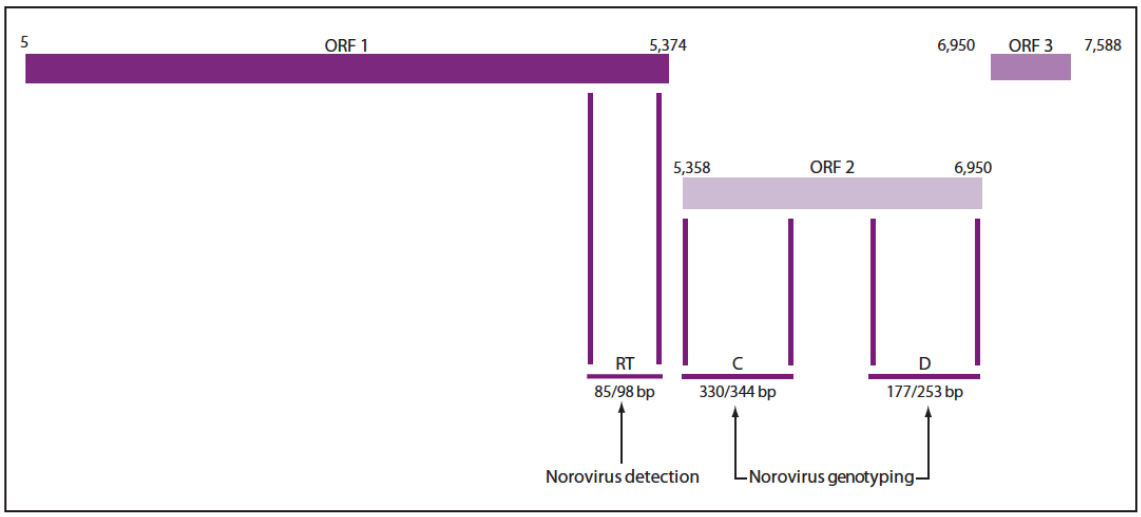
In order to rapidly detect HuNoV in clinical samples, enzyme immunoassays (EIA) have been developed to detect HuNoV antigens. The main advantage of these types of assays is they are easy and quick allowing a large number of samples to be analyzed. The main problem with EIA is they are not broadly reactive and are specific to a certain strain, leading to underreported detection of HuNoV. There are a number of

different HuNoV strains that are antigenically different. In addition, GII.4, the most prevalent cause of human infection, has been shown to evolve rapidly resulting in even greater antigen variation (Hall et al. 2011; Lindesmith et al. 2011; Rabenau et al. 2003). Therefore, EIA may be used as a screening tool but should use other methods for confirmation.

Molecular Techniques

Reverse transcriptase polymerase chain reaction (RT-PCR) as well as Real Time RT PCR (RT-qPCR) are used extensively for detection of HuNoV. PCR-based methods are considered to be extremely sensitive and specific. **Figure 1.6** shows the highly conserved regions of the HuNoV genome targeted by primers and probes. These areas can include the junction of ORF1-2, the RNA dependent RNA polymerase, and the capsid gene region (Kageyama et al. 2003; Knight et al. 2012). Rabenau et al. (2003) SOMETHING IS MISSING HERE investigated the sensitivity of PCR, TEM, and ELISA for detecting HuNoV in a serially diluted clinical sample. RT-PCR was able to detect HuNoV in stool samples diluted to 10^{-4} where ELISA and TEM could only detect at 10^{-2}. This study did not quantify the amount of HuNoV present, however, it did indicate the typical limit of detection for TEM, ELISA, and PCR was 10^{5-7}, $>10^{5}$, and 10^{2} viral particles/ml. One issue with RT-PCR is it requires post amplification steps, such as gel electrophorese and blot hybridization, to detect amplification products. The use of RT-qPCR eliminates this problem. By incorporating fluorescent dyes into the PCR process, nucleic acid amplification can be observed in real time and quantified through the use of standard curves.

Figure 1.6 Genomic areas targeted for detection and genotyping of HuNoV



Abbreviations: ORF = open reading frame; RT = TaqMan real-time RT-PCR region for norovirus detection (*87*); C = region C for norovirus genotyping (*93*); D = region D for norovirus genotyping (*92*); and bp = base pairs.

(Hall et al. 2011) – Hall is not the original author. Cite the original source.

While PCR methods are very sensitive, they too have some drawbacks. PCR techniques allow for amplification of both viable and non-viable nucleic acid. In inactivation studies this has posed an issue as inactivated RNA can still be amplified (Houde et al. 2006). Treatment steps for extracted RNA have been developed to destroy inactivated RNA. These methods have generally involved the use of enzymes, such as proteinase K or RNase (Lamhoujeb et al. 2008; Mormann et al. 2010). Incorporating these enzymes into the PCR process degrades free RNA so that only capsid-associated RNA should be detected. Other drawbacks associated with PCR involve false positive or negative results due to non-specific amplification and PCR inhibitors, respectively. It has been found that by including internal controls, false positive or negative results can be correctly interpreted, and at times indicate where a procedure has gone wrong (Mormann et al. 2010; Rabenau et al. 2003).

Plaque Assay

In order to properly assess the efficacy of inactivation methods, the amount of infectious virus must be quantified. Because molecular techniques cannot distinguish infective virus from non-infectious virus, surrogate viruses are used and detected via plaque assay. This technique allows quantification of infectious virus by counting the lysed zones, or plaques, formed by infecting confluent cell lines with the treated virus. Plaque assay is crucial in determining whether detectable RNA corresponds to the same concentration of infectious virus and can also provide insight into the mechanism of inactivation (Bidawid et al. 2002; Wobus et al. 2004). Inactivation of infectious virus can occur either through damage to the viral RNA, capsid, or both. By comparing the plaque forming units (PFU) and detectable RNA obtained via plaque assay and RT-qPCR, respectively, it may be possible to determine which method of inactivation causes loss of infectivity.

There are disadvantages associated with the plaque assay as well. As opposed to traditional bacterial cultures which can be often be detected in 24 h, the plaque assay takes 48 h incubation plus an additional staining step. Preforming a plaque assay also requires cell culture techniques, which can involve additional steps. Cell cultures require especially aseptic technique as they can be easily contaminated. In addition, whenever chemical disinfection is performed, samples must properly be neutralized before being subjected to plaque assay. The neutralization buffer must be able to not only stop the action of the disinfectant but also ensure the sample is not cytotoxic to the cell culture. If cytoxicity is detected, it must be diluted out which can lead to a lower recovery of virus.

Role of Soft Surfaces as Fomites

Soft Surface Persistence

Studies on soft surface persistence of HuNoV surrogates and other enteric viruses demonstrate that survival on soft surfaces can be influenced by initial resistance to desiccation, environmental conditions, such as temperature and relative humidity (RH), content of the viral medium, fomite type, and exposure method (**Table 1.7**). Lee et al. (2008) demonstrated that MNV could exhibit prolonged survival on gauze and diapers depending on the temperature used for incubation. MNV was detected for up to 40 days when held at -20 **°C** showing minimal reduction in viral titer of <2 and <1 log on gauze and diapers, respectively. Similar results were obtained when held at 4 **°C** exhibiting a 2 log reduction after 30 days on gauze and <2 log reduction after 40 days for diapers. Higher temperatures (18 and 30 **°C**) reduced the survival significantly as a >3 log reduction after one day was observed on both surfaces. Fisher and Shaffer (2010) demonstrated that MS2 could persist at significant levels on coupons excised from a filtering face-piece respirator. Coupons were inoculated with MS2 by liquid and aerosol at an initial concentration of 6.8 and 5.81 log pfu/coupon, respectively, and incubated at 22 °C with 30% RH. They found that MS2 could be recovered at a minimum of 10% of the original titer after 4 days. Additionally they detected MS2 after 10 days with a recoverable titer of 3.2 and 5.7 logs, respectively. Abad et al. (1994) studied the survival of four enteric viruses [human rotavirus (HRV), hepatitis A virus (HAV), poliovirus (PV), and adenovirus (ADV)] on non-porous and porous fomites under different environmental conditions. The results they obtained demonstrated the influence of fomite type as well as environmental conditions would be significantly affected by virus type.

They found that HRV and HAV were significantly more resistant to desiccation (3-5 h at room temperature) exhibiting inactivation rates ranging from 0.1-1.6 logs where as ADV and PV demonstrated inactivation rates ranging from 1.5-4.3 logs. Resistance to desiccation is very important, as it will significantly influence the long-term survival of viruses on surfaces. Inactivation rates were not significantly different due to surface type except for HAV, which exhibited an inactivation of 0.1-0.6 log on non-porous fomites and 1.5-1.6 logs on porous fomites. The persistence of ADV and PV was higher on porous surfaces than non-porous surfaces, however, this effect was not found to be significant. Due to the focus of this review we will focus on the survival of each virus under various conditions for porous surfaces only. When evaluating the effect of temperature and RH, they found that all viruses with the exception of HRV exhibited enhanced survival at 4 **°C** than 20 **°C**. At 4 **°C** reduction in viral titer for HAV, PV, and ADV was >2, >3, and 4 logs, respectively. When assessed at 20 **°C** reduction in viral titer for HAV, PV, and ADC was >3, 5, and 5 logs, respectively. They also evaluated the effect of high RH (80%) (HRH) and moderate RH (50%) (MRH) at 20 **°C**. The effect of RH on porous surfaces was found to be significant only for HRV. This corresponded to a reduction of > 1 log at HRH and >2 logs in viral titer at MRH. The effect of the virus containing medium was also investigated by observing the persistence of each virus suspended in phosphate buffered saline (PBS) as well as 20% fecal suspension (FS). The influence of virus-containing medium is especially important when considering environmental persistence as viruses can often be contained in suspensions containing organic content from either vomit or fecal material. The presence of FS for 4 enteric viruses on porous surfaces was not significant for HRV and HAV, however, it decreased

the survival of PV and ADV on porous surfaces. At 4 **°C** and 90% RH survival of PV and ADV suspended in PBS demonstrated the ability to persist for 30 days exhibiting a decline in viral titer of >3 and >4 logs respectively. When suspended in FS, the survival of both PV and ADV decreased corresponding to 30 days persistence and >5 log reduction in viral titer for PV and only 5 days persistence and a >6 log reduction in viral titer for ADV.

Exposure method is another factor that can influence virus survival. Surfaces can be contaminated through direct contact with bodily fluid by larger droplets or through aerosols generated via vomiting or flushing of toilets. Survival of viruses in aerosols can vary greatly but may have the ability to increase virus survival depending on the composition of the aerosolized media (Lee et al. 2009). Dixon et al. (1966) evaluated the survival of PV at 22 **°C** exposed by direct contact, aerosolization, and dust containing particles on 5 cm swatches of wool and cotton fabrics at both low (35%) and high (78%) relative humidity. Their results demonstrated that the persistence of PV by different methods of exposure could vary significantly due to surface type and RH. At low RH they observed that PV could persist for the longest period of time (20 weeks) on wool blankets exposed to virus by direct contact. On wool gabardine, cotton sheeting, cotton terry, and cotton knit PV demonstrated the ability to persist longest when exposed by aerosol for approximately 10 weeks on wool gabardine and 4-6 weeks on all cotton fabrics. When assessed at high RH wool gabardine demonstrated a longer persistence of PV when exposed to virus by direct contact for 6 weeks, however, on wool blankets the longest persistence was observed for 10 weeks when exposed to aerosols. On all 3 cotton fabrics there was no difference in survival when exposed to direct contact and aerosol (4

weeks). Virus contained in dust was undetectable after 1 week in all experiments, which was attributed to the unstable nature of the virus when lyophilized. The general trend observed, with the exception of the wool blanket, was that viruses in aerosols survived better at low RH whereas virus contaminated through direct contact survived better at high RH. Dixon et al. (1966) observed that at low RH there was an initial rapid decline in viral titer followed by a slower rate of decay than at high RH. The results documented for aerosol survival are different than what has been observed in previous studies which have shown PV contained in aerosol to be more stable at high RH, however, the slower rate of decay of aerosols at low RH has been documented in previous studies (Harper, G.J. 1961; Ijaz et al. 1985). The trends in enhanced survival of viruses applied by direct contact at high RH are typical with respect to PV. Additionally, it may be due to the moisture retained by fabrics, which could protect the virus against the drying effects after the initial absorption of the virus-containing medium. Zuo et al. (2012) also studied the effect of deposition method on the recovery of avian influenza on three non-woven fabrics. They found significant differences in the recovery of AIV deposited by direct contact and through aerosolization. AIV deposited by liquid spiking and aerosol could be recovered between 22-100% and 2-4.4% respectively depending on surface type. The low recovery of aerosolized AIV may have been due to poor survival in aerosols versus liquid but may have also been due to difficulty in recovering aerosol particles. They proposed the aerosolized particles may have been deposited deeper into the fabric substrata making it more difficult to recover. This statement was supported by electron microscopy performed by Lee et al. (2009) who observed that aerosolized MS2 was still present on cotton-polyester filters after extraction by vortexing. The mechanism for inactivation of

virus in aerosols is not completely understood but based on the results of these studies it is clear that the method of deposition can have a significant effect due to recovery or survival.

Table 1.7 Persistence of viruses on soft surfaces including HuNoV and surrogates

Surface/Medium	Virus	Treatment	Significant Results	Reference
Diaper and Gauze	MNV	-20, 4, 18 and 30 °C	-20°C: MNV was detected for up to 40 days exhibiting reduction viral titer of <2 and <1 log on gauze and diapers, respectively. 4°C: MNV exhibited a 2 log reduction after 30 days on gauze and <2 log reduction after 40 days for diapers. 18 and 30°C: MNV was reduced >3 log in viral titer after one day both surfaces	Lee et al. (2008)
Filtering facepiece respirator	MS2	22°C at 30% RH Aerosol and direct contact	MS2 could be recovered at a minimum of 10% of the original titer after 4 days. MS2 was detected after 10 days with a recoverable titer of 3.2 and 5.7 log by aerosol and direct contact.	Fisher and Shaffer. (2010)
Aluminum China Glazed tile Latex Polystyrene Cotton Paper	HAV HRV AD PV	20% fecal suspension or PBS 4 °C and 90% RH 20°C, 95 and 85% RH	General: Virus persisted up to 60 days under ideal conditions No significant difference in survival based on fomite except for HRV Survival better at 4°C than 20°C High RH increased survival Presence of fecal matter generally increased persistence except for PV and ADV on soft surfaces	Abad et al. (1997)

China Paper	AsV	20% fecal suspension or PBS 4 and 20°C 90% RH	4°C: AsV persisted for up to 60 days on China displaying a 4 and 5.3 log reduction in titer when contained in 20% FS or PBS. AsV was able to persist up to 90 days on paper displaying a 4.3 and 4.5 log reduction in viral titer when contained in 20% FS or PBS. 20°C: C the prescience of AsV was undetectable after 7 days w/ or w/o FS except AsV contained in PBS which persisted for up to 60 days on paper displaying an approximately 4 log reduction. When assessing the persistence of AsV once a day for a total of 7 days they found that AsV persistence was increased on paper in the presence of FS at both 4 and 20 °C.	Abad et al. (2001)
Cotton and wool fabrics: Wool blanket Wool gabardine Cotton sheeting Cotton terry cloth Cotton jersey knit	Vaccinia	Exposed via direct contact, aerosol, and virus containing dust, 35 and 78% RH 25 °C	35% RH: Vaccinia could be recovered up 14 weeks on wool blankets and 16 weeks on wool gabardine when inoculated by dust and direct contact, respectively. On all cotton fabrics Vaccinia survived 10-15 weeks when inoculated in dust containing particles. 78% RH: On wool blanketing Vaccinia persisted for 6 weeks regardless of deposition method however on wool gabardine aerosols persisted up to 10 weeks. On cotton sheeting and cotton terry Vaccinia persisted for 6 weeks and 4 weeks, respectively, when contained in dust or aerosol. On cotton knit jersey the virus persisted for 6 weeks	Sidwell et al. (1966)

Cotton and wool fabrics: Wool blanket Wool gabardine Cotton sheeting Cotton terry cloth Cotton jersey knit	Poliovirus	Exposed via direct contact, aerosol, and virus containing dust, 35 and 78% RH 25 °C	At 35% RH up to 20 weeks on wool and 1-4 week on cotton fabric At 35% RH wool titers decreased rapidly but persisted longer AT 78% RH decline was less rapid but didn't persist as long Cotton at both RH had rapid decrease in titer Dust containing particles survived shortest amount of time.	Dixon et al. (1966)

Soft Surface Transmission

Evidence of HuNoV transmission from soft surfaces comes mainly from epidemiological evidence, however, there are several investigative studies, which demonstrate the ability of viruses to be transmitted from various types of soft surfaces (**Table 1.8)**. Gibson et al. (2012) investigated the transfer of FCV, MNV, MS2, and PRD1 from porous surfaces to non-porous surfaces under laboratory conditions. A viral cocktail containing all 4 surrogates at approximately 5-6 log pfu/ml was applied to 5 cm^2 swatches of two cellulose/cotton cloths, one microfiber cloth, one nonwoven cloth, and one cotton terry towel. After 1 min to allow for full saturation of the swatches they were used to wipe 7.6 cm^2 coupons of stainless steel or non-porous acrylic. They found that there was a total average of 0.53, 0.92, 2.51, and 2.91 log pfu/ml transfer to non-porous acrylic by cellulose/cotton cloths, microfiber cloth, nonwoven cloth, and cotton terry towel, respectively. Total transfer of virus to stainless steel was 0.41, >1, 1, 2.5, and 2.06 log pfu/ml from cellulose/cotton cloth 1, cellulose cotton cloth 2, microfiber cloth, nonwoven cloth, and cotton terry towel, respectively. The transfer of viruses by cleaning cloths was found to vary significantly by cloth type. Lopez et al. (2013) investigated the transfer of bacteria and viruses from porous and non-porous fomites to fingers under low (15-32%) and high (40-65%) relative humidity. MS2 was inoculated to a 1 cm^2 portion of cotton, polyester, and paper currency at a concentration of 9-11 log pfu/cm^2 and allowed to dry for 30 min. Fomite to finger transfer was assessed after the index, middle, and ring finger were pressed to fomites in three separate events. The authors observed that at low relative humidity the transfer efficiency of MS2 from cotton, polyester, and paper currency was 0.03, 0.3, and 0.4%, respectively. Under high relative humidity conditions

the transfer efficiency of MS2 was higher resulting in 0.3, 2.3, and 0,7%, respectively,, however, only the transfer from polyester was significantly different. With a starting inoculum of 10^9-10^{10} pfu/ml, this means that there is the potential for approximately 10^5 pfu/ml to be transferred to hands from porous surfaces. O'Toole et al. (2009) evaluated the transfer of MS2 to hands from 100 cm^2 swatches of 65/35 cotton/poly blended knit weave, 100% cotton toweling, and 100% cotton knit weave. After washing swatches in contaminated wash water they found there was an average of 3.77 log pfu/swatch of MS2. They further demonstrated that 1.01 log pfu/swatch could be subsequently transferred to fingertips resulting in an average transfer rate of 0.19%. Sidewell et al. (1970) also evaluated the transmission of Poliovirus (PV) and Vaccinia virus (VV) by dry contact fabrics. PV and VV were applied to sterile swatches of wool and cotton fabrics by direct contact and aerosolization and allowed to dry for 16 h at 25° C and 35% RH. After drying, contaminated swatches were manually tumbled with dry sterile fabrics and assessed from 1-30 min to determine the maximum transfer of virus to sterile fabrics. Wool blanketing was found to transfer virus with the greatest ease resulting in maximum transfer of virus applied by direct contact and aerosol of 3.5 and 2.8 logs after 20 and 3 min for PV and 4.4 and 4.2 log after 10 and 20 min for VV, respectively. The lowest transfer was observed for PV applied by direct contact and aerosol from Dacron/cotton shirting by 0.4 and 0.6 log after 20 and 10 min, respectively. The lowest transfer observed for VV was by direct contact applied to cotton sheeting and washable wool shirting by 0.6 and 1.8 logs after 10 min, respectively. They also found that PV generally transferred with greatest ease when applied to fabrics by aerosol though VV generally transferred with greater ease than PV regardless of deposition method. Gerba and

Kennedy (2007) evaluated the transfer of Rotavirus (RV), Hepatitis A virus (HAV), and Adenovirus (ADV) from inoculated cotton swatches to sterile swatches during laundering practices. In separate experiments, 4 cotton swatches were inoculated with 6.52, 6.42, and 5.19 log pfu/ml RV, HAV, and ADV respectively. After drying for 30 min swatches were washed with detergent using a 12/3 min wash/rinse cycle at 20-23 **°C**. The swatches were washed with 4 sterile cotton swatches for which the transfer rate was determined along with 3.2 kg of sterile cotton clothing consisting of t-shirts and underwear. To simulate a realistic organic load, which may be present during laundering, 1 pillowcase containing 31.2 g sebum was also included. After washing, swatches were allowed to sit in the washer for 30 min followed by tumble-drying for 28 min during which the fabrics reached a temperature of 55 **°C**. The results of this experiment showed that RV, HAV, and ADV could transfer 3.54, 3.18, and 3.4 log pfu/swatch, respectively, to sterile swatches during washing. After drying, the transfer of RV, HAV, and ADV was determined to be 3.35, 3.43, and 3.4 log pfu/swatch, respectively. This study demonstrates that contaminated fabrics washed and dried using a cold-water setting and detergent alone would allow for significant amount of viruses to be transferred to sterile fabrics. This could result in further transfer of viruses to hands during the handling of fabric during laundering. Though the amount of virus transferred from soft surfaces in these experiments varied from low to high, it is important to remember that only 10-100 viral particles of HuNoV are needed to cause illness.

Table 1.8 Transmission of viruses on soft surfaces including HuNoV and surrogates

Surface/Medium	Virus	Treatment	Significant Results	Reference
Cleaning cloths: two cellulose/cotton cloths, one microfiber cloth, one nonwoven cloth, and one cotton terry towel	MNV FCV PRD1 MS2	A viral cocktail at approximately 10^5-10^6 pfu/ml was applied to 5 cm^2 swatches cleaning cloths. After 1 min to allow for full saturation of the swatches they were used to wipe 7.6 cm^2 coupons of stainless steel or non-porous acrylic	FCV, MS2, and PRD1 resulted in a total average transfer of 0.53, 0.92, 2.51, and 2.91 log pfu/ml to non-porous acrylic by cellulose/cotton cloths, microfiber cloth, nonwoven cloth, and cotton terry towel. Average total transfer of virus to stainless steel was 0.41, >1, 1, 2.5, and 2.06 log pfu/ml from cellulose/cotton cloth 1, cellulose cotton cloth 2, microfiber, cloth, nonwoven cloth, and cotton terry towel	Gibson et al. (2012)
Cotton Polyester Paper currency	MS2	1 cm^2 portion of cotton, polyester, and paper currency inculcated at at a concentration of 10^9-10^{11} pfu/cm^2 and dried for 30 min. Index, middle, and ring finger were pressed to fomites at 15-32%, 40-65% RH	15-32% RH: transfer efficiency of MS2 from cotton, polyester, and paper currency was 0.03, 0.3, and 0.4%, respectively. 40-65% RH: ransfer efficiency of MS2 was higher resulting in 0.3, 2.3, and 0,7%, respectively, however only the transfer from polyester was significantly different.	Lopez et al. (2013)

Hands 65/35 cotton/polyester blended knit weave 100% cotton toweling 100% cotton knit weave	MS2	100 cm^2 swatches washed in 69 L contaminated wash water suing 16/10 min rinse/spin cycle	An average of 3.77 log pfu/swatch of MS2 was found on swatches after washing, of which 1.01 log pfu/swatch could be subsequently transferred to fingertips resulting in an average transfer rate of 0.19%	O'toole et al. (2009)
Cotton and wool fabrics: Wool blanket Washable wool shirting Cotton sheeting Cotton terry cloth Nylon jersey Dacron/Cotton Shirting	Poliovirus Vaccinia Virus	Virus deposited by direct contact and aerosol for16 h at 25 °C 35% RH After incubation swatches were tumbled with sterile swatches	10^3 poliovirus and 10^4 vaccina transferred to sterile swatches within 1-10 minutes Maximum transferred by wool. Poliovirus aerosol transferred easier than direct contact.	Sidwell et al. (1970)
Cotton and wool fabrics: Wool blanket Washable wool shirting Cotton sheeting Cotton terry cloth Dull nylon jersey Dacron/Cotton Shirting	Poliovirus	Fabrics inoculated by aerosol or direct contact Washed with anionic and nonionic detergents in 44 L of 21-27, 38-43, and 54-60°C	Rate of transfer in all trials was not significantly different resulting in an average 1.46 log CCID50 poliovirus transferred to sterile swatches	Sidwell et al. (1971)
Cotton swatches	RV HAV ADV	Swatches inoculated with 6.52, 6.42, and 5.19 log pfu/ml RV, HAV, and ADV and washed with detergent using a	RV, HAV, and ADV could transfer 3.54, 3.18, and 3.4 log pfu/swatch, respectively, to sterile swatches during washing. After drying the transfer of RV, HAV, and ADV was determined to be 3.35, 3.43, and 3.4 log pfu/swatch, respectively	Gerba and Kennedy. (2007)

	12/3 min wash/rinse cycle at 20-23°C and dried 28 min reaching 55°C	

Supporting Epidemiological Evidence

As HuNoV cannot be cultivated *in vitro,* the best examples of soft surfaces as disseminators for HuNoV come from epidemiological evidence. This evidence highlights factors that make HuNoV a risk for environmental transmission, such as the ability to persist in the environment as well as resist typical forms of decontamination. The evidence also demonstrates the role soft surfaces themselves play as significant fomites that are difficult to decontaminate.

Case Study #1

One early example is a case where two carpet fitters became ill after removing carpeting from a ward where multiple cases of gastroenteritis due to HuNoV, were documented (Chessbrough et al. 1997). The final case of the 5 day outbreak occurred 16 days prior to the presence of the workers. Cleaning of the ward consisted of double wiping of hard surfaces and dry vacuuming of the carpet 12 days prior to the arrival of the workers. Vacuuming of the carpeting continued daily. Despite the cleaning procedures both workers became infected, displaying symptoms of gastroenteritis within 36-48 h. While no further epidemiological investigation was done to determine the source of the infection, the increased contact with the carpet versus other surfaces suggests the contaminated carpet was the source. If the carpet was the source of these two cases, then it would indicate that not only can HuNoV survive at least 16 days in the environment but also that repeated dry vacuuming is an insufficient method to remove it from carpets.

Case Study #2

Another investigation by Chessbrough et al. (2000) further highlights the persistence of HuNoV and transmission through the environment as well as the difficulty in cleaning soft surfaces especially carpets. From January to May 1996, a prolonged outbreak of HuNoV occurred at a large hotel. The initial outbreak affected 850 guests over a 12 week period. The hotel closed on March 15th to undergo "thorough" cleaning which, included cleaning hard surfaces with detergents and warm water as well as shampooing and vacuuming carpets. It was noted that disinfectants were specifically not used due to concerns on the effect on the quality of carpets and other soft surfaces. Cases of HuNoV appeared rapidly upon reopening 1 week later, peaking at 92 cases by April 1st.

Many factors contribute to an outbreak this large with numerous possible routes of transmission. It is hard to pinpoint exactly how HuNoV was transmitted but epidemiological evidence suggest environmental transmission played a large role. Through the course of the investigation, no food items were identified as a possible source of the outbreak, there were no lapses in hygiene in the kitchen or among the staff, indicating that food or water were not the source of the ongoing contamination. In order to reduce the potential of direct person-to person-transmission, cohorts of guests were kept separate when they were arriving or departing yet this had no effect on the course of the outbreak. Environmental samples tested via nested RT-PCR revealed that many different surfaces, including hard and soft surfaces, tested positive for HuNoV even after cleaning. Contaminated surfaces would explain the infection of guest in different groups and the ability of the outbreak to resume after a week of the hotel being closed. Carpet

samples showed the highest percent of positive samples (75%) even after cleaning with warm water and detergents as well as daily vacuuming. Though it is unclear precisely how HuNoV could be transmitted from the carpet, reaerosolization of surface bound HuNoV is one proposed method.

Case Study #3

Evans et al. (2002) reported a HuNoV outbreak at a concert hall that affected 300 people over a 5 day period. The factors that contributed to this outbreak were similar to the last two case studies. After thorough investigation the source of the outbreak was traced back to one individual seated in tier 13 who vomited in several areas including a bathroom, waste bin, emergency exit, and carpeted corridor leading to the stairs. All surfaces were cleaned with an "emergency spillage compound" and carpets were also vacuumed. The following day, 1229 children attended a concert from 15 different school groups. Within 24-48 h, 257 children reported symptoms of gastrointestinal illness. Fecal samples taken from the ill students were positive for HuNoV. Epidemiological investigation was undertaken and revealed that no foods or drink were linked to the infection. It was determined that contaminated fomites were most likely the cause of the outbreak. The highest attack rate (75%) was observed for those seated in tier 13. In addition attack rates were higher (30-50%) for those students who exited via the carpeted corridor and lower (≥ 10%) for those who did not use the carpeted corridor. In addition, due to the fact that the first vomiting incident occurred 24 h prior to the schools arrival the concert hall, direct person-to-person transmission could not be possible.

All outbreaks summarized in **Table 1.9** provide strong evidence that HuNoV can persist on and be transmitted through soft surfaces. In most cases outbreaks of HuNoV have persisted due to cleaning with non-hypochlorite disinfectants. Soft surfaces are especially problematic for decontamination due to their complex structure and susceptibly to qualitative changes. In outbreaks where soft surfaces have been implicated, soft surfaces have always received the least stringent method of decontamination, generally vacuuming and/or a wash step with warm water and detergents. Shampooing and vacuuming are clearly not sufficient for decontamination and there is a need for more thorough disinfection protocols and studies.

Table 1.9 Epidemiological evidence of soft surfaces as fomites for HuNoV

Setting	Surface	Infected cases	Duration of Outbreak	Disinfection methods	Implications	Reference
Concert Hall	Carpet	>300	5 days	"emergency spillage compound", vacuuming	High attack rate (30-50%) associated with use of carpeted corridor	Evans, M.R. et al. (2002)
Airplane	Carpet, Upholstered seats, curtains	27	5 days	Soft surfaces within 3 rows on incident were removed. Other carpeted areas received steam cleaning	Only hard surfaces were swabbed which came back negative for Norovirus. Suggest HuNoV could have persisted on carpets after steam cleaning	Thornley, C. et al. (2011)
Hotel	Carpet	942	5 months	Vacuuming, water, detergents	62% carpets tested positive after cleaning	Chessbrough, J. et al. (2000)
Hospital	Carpet	2	N/A	Vacuuming	HuNoV was contracted while removing carpets 16 days after last exposure	Chessbrough, J. et al. (1997)
Soccer Tournament	Reusable grocery bag	10	N/A	N/A	Soft surface contaminated via aerosol, transferred to other surfaces	Repp, K. et al. (2011)

Challenges to the Study of Soft Porous Surfaces

Intrinsic Properties of Soft Surfaces

The porous nature of soft surfaces is what distinguishes them from hard non-porous surfaces and contributes to their role as fomites. The interaction of liquids with soft surfaces is mainly described by the wettability, moisture retention, and moisture regain of fibers (Hsieh et al. 1992; Hsieh and Timm, 1987). Wettability is measured by the time it takes for a surface to absorb a liquid and the time it takes the liquid to wick, or travel a certain distance along the surface. Moisture retention is the amount of liquid that a fully saturated fiber can retain whereas moisture regain is the amount of moisture absorbed from the air under ambient conditions and is used to determine hydrophobicity. Hydrophobicity also influences the wettability as surfaces with lower hydrophobicity have shorter absorbance and wicking times (Hsieh and Timm, 1987; Weaver, J. W. 1984). Fabrics exhibit a large variation in liquid-surface interactions due to the many diverse fiber types (natural, synthetic, blended), however, fabrication (weave, knit, woven) will also influence these interactions. Studies on the wettability and retention properties of single fibers and woven fabrics have shown that fabrics and fibers composed of the same materials will demonstrate similar wettability due to adsorption regardless of their construction. The wettability due to wicking, and the retention of liquid by fabrics of the same fiber type, however, will vary based on fabrication due to differences in the geometry of the pore structure created in the substrata of the fabric as well as the wettability of the fibers (Hsieh et al.1992).

Liquid-surface interactions are important to understand as they may influence the attachment, survival, and recovery of viruses on soft surfaces. Hydrophilic surfaces may

allow for increased virus binding as compared to hydrophobic surfaces due to the effect on wettability. When liquid is applied to hydrophobic surfaces, it forms a droplet, which is slowly absorbed, across a small distance. Liquid applied to hydrophilic surfaces, however, is absorbed quickly and disperse over a large area allowing for a greater surface area for viral attachment making them harder to recover (Zuo et al. 2013). Surface type may also have an influence on persistence of viruses on soft surfaces. Viruses have demonstrated the ability to persist for different amounts of time on fabrics of different fiber types as well as fabrics of the same fiber type with different fabrications. Differences in persistence may be due some protective effects of the fabrics, such as high natural moisture content or moisture retention (Dixon et al. 1966; Sidwell et al. 1966; Sidwell et al. 1970). Examples of soft surface transmission and persistence will be discussed in greater detail later on in this review.

Fiber type and fabrication can also have an effect on the efficacy of disinfection. Differences in fabrication will affect the concentration of a disinfectant that can be removed from solution. McNeil et al. (1960) demonstrated that a single piece of yarn constructed of either muslin or gauze adsorbed 20.8 and 21.1 mg/L, respectively, of quaternary ammonium compound (QUAT). When these yarns were woven into fabric, however, the adsorption changed. When woven into fabric, muslin only adsorbed 8.2 mg/L and gauze absorbed 21.6 mg/L. This result was attributed to a larger surface area in the loose knit gauze. Adsorption of active ingredient from solution is of importance because it changes the amount of active ingredient available for disinfection. This effect was demonstrated by Goldsmith et al. (1954) who studied the disinfection of cotton and wool contaminated with *E. coli* and *Micrococcus pyogenes* var. *aureus* 209. On cotton

and wool the concentration for inactivation using a QUAT ranged from 200-2000 ppm and 100-600 ppm, respectively. For disinfection with sodium hypochlorite a concentration of 8-20 ppm and 40-800 ppm were needed for cotton and wool, respectively. Cotton needed a higher concentration of QUAT (2000 ppm) whereas wool needed a higher concentration of sodium hypochlorite (800 ppm). Differences in concentration are attributed to the reaction of the disinfectants with naturally occurring constituents of the fabric such as cellulose or keratin. Cotton contains cellulose, which is able to rapidly inactivate QUAT at concentrations up to 500 ppm at which <10% can be removed from solution. Wool on the other hand contains keratin, which will inactivate sodium hypochlorite, removing 90% from low concentrations and up to 98% from solutions containing 800 ppm. These results indicate that in order to achieve inactivation of pathogens the absorptive capacity of the fabrics must first be met. As the absorptive capacities of each fabric may be different, special care should be taken when determining the concentrations needed for disinfection.

Soft/Porous Surface Sampling

As previously stated swabbing has been shown to recover HuNoV from multiple surface types including soft/ porous surfaces such as carpets, lampshades, fingers, and food surfaces. The efficacy of swabbing, however, can vary greatly depending on surface type. Scherer et al. (2009) documented a wide range of recovery efficiencies from 2-78% when swabbing foods and food contact surfaces. Lowest rates of recovery (2 and 10%) were associated with porous surfaces of wood and ham. Studies evaluating the recovery of bacteria from textiles indicate that the recovery from fabric by swabbing can be as low

as 0.001% (Rabuza et al. 2012). Swabbing relies on adsorption of the virus to the swab followed by an elution method to either release the virus or extract the RNA (Verran et al. 2010). Due to the low recovery rate sometimes associated with swabbing, agitation-elution methods are often used. Agitation-elution methods rely on physical methods to enhance the removal of pathogens directly from the surface. Taku et al. (2002) demonstrated that agitation-elution using scraping and aspiration recovered more virus from stainless steel (71%) than with cotton swabs or nylon filters (10 and 23%). Fino and Kniel (2008) demonstrated that agitation-elution could increase recovery from food surfaces. In their study they found that FCV could be recovered from fresh produce (strawberry, lettuce, and green onion) at 75-87.5% efficiency, which was comparable or better to the performance of swabbing on similar surfaces.

Agitation-elution methods, sometimes referred to as destructive sampling, are especially important when recovering pathogens from soft porous surfaces as these typically yield the lowest rate of recovery. Rabuza et al. (2012) demonstrated that agitation-elution by shaking for 10 min at 300 rpm recovered 100 to 1000X more bacteria from textiles than swabbing and impression plating. Agitation-elution methods that have been successfully implemented include shaking, vortexing, stomaching, and sonication. Even though agitation-elution methods are designed to enhance recovery, the recovery of pathogens from soft surfaces is still typically lower than from hard surfaces. Gibson et al. (2012) demonstrated that swabbing of hard surfaces could recover FCV at 57% whereas agitation-elution of cleaning cloths by shaking for 30 min at 150 rpm resulted in a 36% recovery. While lower recovery is documented from cleaning cloths, a recovery efficiency of 36% is higher than has been documented with swabbing from similar

surfaces. It is also important to note that the efficiency of recovery will depend on the virus. Gibson et al. (2012) evaluated the recovery of MS2 and MNV in addition to FCV. While the recovery of FCV varied by surface type MS2 showed a >100% recovery from both hard and soft surfaces. MNV had a recovery rate of 41% from hard surfaces, however, the recovery from soft surfaces could not be determined due to low pfu in recovered samples. As different types of soft surfaces may exhibit different affinities for different viruses, further investigation is needed to determine what method will result in maximum recovery efficiency.

Inactivation of Viruses on Soft Porous Surfaces

In the above-mentioned case studies, outbreaks of HuNoV were able to persist in part due to the ineffective decontamination of carpets. Cleaning with 5,000 ppm bleach is the current method recommended for surface disinfection, however, due to the harmful effect that bleach can have on some fabrics, its use on soft surfaces in the environment is impractical. In each outbreak soft surfaces were cleaned by vacuuming alone or in combination with a non-sodium hypochlorite chemical agent, which was ineffective against HuNoV. Issues concerning decontamination of soft surfaces can be attributed to the fact that soft surface disinfection studies are sparse and there are no specific guidelines for cleaning non-launderable soft surface surfaces.

Table 1.10 summarizes disinfection studies for viruses on soft surfaces including HuNoV and its surrogates. Hudson et al. (2007) investigated the use of 20-25 ppm ozone gas for 20 min to inactivate FCV and HuNoV dried onto polystyrene plastic, cotton tips, fabric, and carpet placed in various locations in a 34 m^3 office. They demonstrated that

ozone gas was capable of achieving 3.52-4.52 log reduction in viral titer of FCV and 108.65-112.12 ng reduction in HuNoV RNA on all surface types. There were no significant differences observed due to surface type or location in the office space. Malik et al. (2006) investigated the efficacy of 5 sanitizers on both cotton and polyester fabrics as well as blended carpet. Only one sanitizer, 2.6% activated glutaraldehyde, was able to achieve at least 99.9% reduction in the titer of FCV on all surfaces within 1-5 min with the exception of blended carpet which achieved a 99% reduction after 10 min. Efficacy of most disinfectants increased with contact time except on blended carpets where decreased inactivation was found after 5 and 10 min versus 1 min for 3 of the 5 disinfectants tested. They also observed that fabrics were easier to disinfect than carpets with the exception of 100% polyester fabric, which was the least susceptible to disinfection except by 2.6%, activated glutaraldehyde. Results from this study contradict results found by Gulati et al. (2001) which found that FCV was inactivated >4 logs by phenolic compounds on stainless steel., however, Malik et al. (2006) found that on fabrics and carpets the same phenolic compound achieved a maximum reduction of < 2 log in viral titer.

Differences between inactivation of non-porous and porous surfaces were also documented by Tuladhar et al. (2010). When vaporous H_20_2 was applied to MNV on stainless steel an inactivation of >4 log was achieved. However, when applied to gauze, only a 3 log reduction in viral titer of MNV was observed. Differences in efficacy due to surface type are often attributed to irreversible binding of virus to fabrics which may make it difficult to document similar levels of inactivation, however, it may also be due to interaction between the disinfectant and surface resulting in the removal of the active ingredient from solution (Malik et al. 2006; Tiwari et al. 2006). Additionally viruses

bound to surfaces may be more resistant to inactivation through the formation of aggregates or by occupying binding sites that may be essential for antiviral action (Sobsey and Meschke. 2003). This effect could be enhanced on soft surfaces to which viruses may become more strongly attached to the surface or within the subsurface.

Though studies of environmental decontamination of soft surfaces are limited, the disinfection of viruses on fabrics during laundering has been extensively studied. Sidwell et al. (1970) investigated the efficacy of detergent-disinfectant combinations used in laundering practices at inactivating Poliovirus and Vaccinia virus inoculated onto woolskin bedpads by aerosol and direct contact. They evaluated the efficacy of washing with water, anionic and nonioinic detergents, as well as detergents in combination with alkalinized glutaraldehyde, quaternary ammonium, and phenolic disinfectants. Woolskin bedpads were inculcated with Poliovirus and Vaccinia virus by direct contact or aerosol and laundered at a temperature of 50 **°C** for 10 min followed by a 3 min rinse cycle at 39 **°C** and finally a 6 min spin dry cycle. The authors found no significant difference in the inactivation achieved by detergent type or method of virus deposition. They found that all laundering methods achieved significant reductions of Vaccinia virus, however, only the glutaraldehyde based disinfectant in combination with detergents was able to completely inactivate Vaccinia virus by >4.9 logs and >4.4 logs when applied by direct contact and aerosol, respectively. Poliovirus was still detected in all trials, however, disinfection with detergents and glutaraldehyde resulted in 5.3 and 4.0 log reduction when applied by direct contact and aerosol, respectively. When evaluating the rinse water for the presence of virus they observed that no Vaccinia could be recovered, however, up to 3 log PFU of Poliovirus could be recovered in some trial indicating that reductions in viral titer could

have been partially due to elution by the laundering process rather than inactivation. The fact that washing with water alone was able to achieve significant reductions in the viral titer was attributed to physical factors associated with washing, such as the volume and temperature of the wash water. While glutaraldehyde appeared to be the most effective method for virus inactivation, WHO?they did note that at high concentration the disinfectant caused unfavorable effects on the wool fabric presumably caused by precipitation of the detergent onto the fabric. Sidwell et al. (1971) further evaluated the effects of laundering on several other types of fabrics contaminated with Poliovirus. Cotton sheeting, cotton terry, washable wool shirting, wool blanket, dull nylon jersey, and Dacron/cotton shirting were cut into either 35 x 105 cm strips or 5 cm diameter swatches and inoculated with poliovirus by direct contact or aerosol. The swatches were laundered with anionic or nonionic detergents in 44 L of water at temperatures of 21-27 (cold), 38-43 (warm), or 56-60 **°C** (hot). They found that while detergent type made little difference in the observed reduction, hot water achieved significantly more reduction in viral titer than either warm or cold water and tended to inactivate viruses more easily when inoculated by direct contact than aerosol. Additionally they observed that in a majority of cases the surface type did not influence inactivation, however, Poliovirus was eliminated with greater ease on nylon jersey and was able to persist for longer during drying on wool blanketing laundered in warm water with anionic detergent. The average reduction in viral titer of poliovirus by laundering with detergent on all surface types inoculated by direct contact and aerosol was 5 and 4.2, 3.7 and 2.5, and 3.3 and 2.04 log $CCID_{50}$/ml in hot, warm, and cold water, respectively. Though the reduction in viral titer could be attributed to either inactivation or elution from the fabrics the absence of

poliovirus in the wash water in hot water experiments indicate that in this case the virus was successfully inactivated. When evaluating the presence of poliovirus in wash water in warm and cold water experiments, there was a large variation due to surface type, however, in some cases poliovirus was present in titer of up to 3.9 logs. The presence of Poliovirus in the wash water when using cold water was only apparent when washing wool fabrics. In warm water the washing of both wool fabrics and cotton terry cloth resulted in the presence of Poliovirus in the wash water. This indicates that while laundering may be effective at reducing contamination on fabrics there may still be a great chance for transmission to sterile fabrics during laundering.

Washing with detergents alone have previously been shown to be ineffective at inactivating viruses,, however, new technologies being used to develop detergents may make them more effective. Hienzel et al. (2010) investigated the effect of laundering with a peracetic acid-based detergent against Poliovirus. In this study the authors compared the efficacy of washing with either tap water alone or 0.8% Persil Meapearls. Poliovirus was inoculated onto cotton swatches at approximately 7.98 logs/swatch and washed for 1 h at 30 **°C** with sterile terry towels. They observed that1 h of washing in tap water resulted in reduction of 2.68 logs/swatch with approximately 4.5 log/ml found in the wash water, When washing with 0.8% detergent they found that PV was completely inactivated below the limit of detection on swatches as well as in the wash water. As compared to previously discussed studies this indicates that virus is not only being removed from swatches but completely inactivated during the washing process through the use of the pearceitic acid based detergent.

Another way to reduce this risk of transmission during laundering practices is to incorporate more effective disinfectants into the washing procedure. Gerba and Kennedy (2007) evaluated the use of detergents and sodium hypochlorite at inactivating ADV, HAV, and Rotavirus contaminated clothing during laundering procedures. Cotton swatches were inoculated with either 6.52, 6.42, and 5.19 log pfu/ml of RV, HAV, and ADV, respectively, and washed with detergent alone or detergent with 1 cup of 5.25% sodium hypochlorite resulting in free chlorine concentrations of approximately 114-125 ppm. The swatches were washed using a 12/3 min wash/rinse cycle at 20-23 **°C** with 4 sterile cotton swatches, 3.2 kg of sterile cotton clothing, as well as 1 pillowcase containing an organic load of 31.2 g sebum After washing, swatches were allowed to sit in the washer for 30 min followed by tumble-drying for 28 min during which the fabrics reached a temperature of 55 **°C**. The authors observed that washing with detergent alone achieved 2.88, 2.74, and 1.1 log reduction in viral titer of RV, HAV, and ADV, respectively. When washing with detergent and bleach RV, HAV, and ADV achieved a 5.82, 6.48, and 4.09 log reduction in viral titer, respectively. Drying of fabrics was also shown to further increase the levels of inactivation. After washing with detergent alone and in combination with bleach, drying of swatches achieved a total of 3.2, 3.03, 2.47 and 6.88, 6.58, and 4.38 log reduction in RV, HAV, and ADV, respectively.

Another method for reducing transmission of viruses by fabrics is to use antimicrobial textiles. Sidewell et al. (1967) investigated the effect of fabrics impregnated with antimicrobials on the persistence of vaccine virus and poliovirus. Wool blanketing, wool gabardine, and cotton sheeting impregnated with a QUAT were inoculated with Vaccinia virus and evaluated for persistence at 25 **°C** at 35 and 78% RH.

In that study they found that impregnated fabrics inactivated Vaccinia virus by >4 log after only 30 min. They further investigated the virucidal effect of cotton fabrics with a "wash and wear" finish modified with triazone. They found that Vaccinia persisted on wash and wear fabrics for less than 1 day, however, poliovirus persisted for up to 5 days. As these fabrics were already modified the persistence could not compared to untreated fabrics as with the QUAT impregnated fabrics

Table 1.10 Inactivation of viruses on soft surfaces including HuNoV and surrogates

Surface	Virus	Treatment	Significant Results	Reference
Office and hotel rooms were used Surfaces included plastic, fabric, cotton, and carpet	FCV HuNoV	20-25 ppm ozone with 5 min vapor burst 10 min incubation	Greater than 3 log reduction in infectivity for FCV and RNA for HuNoV	Hudson et al. (2007)
Fabrics: Cotton Polyester, Cotton Polyester blend Carpets: olefin, polyester, nylon/olefn blend	FCV	Treated with 5 different disinfectant solutions for 1-10 min	Only 1 disinfectant capable of achieving 99.9% reduction on all surfaces Blended carpet could not be disinfected Polyester least amenable	Malik et al. (2006)
Stainless steel Framing panel Gauze	HuNoV.GII MNV	Hydrogen peroxide vapor 127 ppm 1 hr	>4 and 3 log reduction of MNV on stainless steel and gauze respectively 0.5 log reduction of HuNoV.GII RNA on stainless steel	Tuladhar et al. (2012)
Wool blanketing Wool sheeting Cotton sheeting Cotton "wash-and wear" with triazone resing	Vaccinia virus Poliovirus	Wool and non "wash-and-wear" cotton fabrics were impregnated with QUAT and inoculated with Vaccinia Virus and Poliovirus. Cotton "wash-and wear" were inoculated with both viruses. Fabrics were assessed at 25°C at 35 and 78% RH	Impregnated fabrics inactivated Vaccinia virus by >4 log after only 30 min On cotton "wash and wear" Vaccinia and Poliovirus persisted for less than 1 day and up to 5 days, respectively	Sidwell et al. (1967)

Cotton swatches	RV HAV ADV	Swatches inoculated with 6.52, 6.42, and 5.19 log pfu/ml RV, HAV, and ADV and washed with detergent alone or with 114-125 ppm bleach using a 12/3 min wash/rinse cycle at 20-23°C and dried 28 min reaching 55°C	Detergent alone achieved 2.88, 2.74, and 1.1 log reduction in viral titer of RTV, HAV, and ADV, respectively. Washing with detergent and bleach RV, HAV, and ADV achieved a 5.82, 6.48, and 4.09 log reduction in viral titer. Drying of fabrics was also shown to increase the levels of inactivation in when washing with detergent alone and in combination with bleach resulting in a total of 3.2, 3.03, 2.47 and 6.88, 6.58, and 4.38 log reduction in RV,HAV, and ADV, respectively.	Gerba and Kennedy et al. (2007)
Cotton	Poliovirus	Swatches with 7.98 log/swatch Poliovirus received 1 h washing in 9.20 L tap water at 30.7-31.6°C Washing with tap water alone or 0.8% Persil Megapearls	1 h of washing in tap water resulted in reduction of 2.68 log/swatch with approximately 4.5 log/ml found in the wash water. Washing with 0.8% detergent resulted in complete inactivation of PV below the limit of detection on swatches as well as in the wash water	Heinzel et al. (2010)

Glutaraldehyde-tanned woolskin bed pads	Vaccinia virus Poliovirus	Woolskin bedpads were inoculated with poliovirus and vaccinia virus by direct contact or aerosol and laundered at a temperature of 50 C for 10 min followed by a 3 min rinse cycle at 39 C and finally a 6 min spin dry cycle. Washing was done with water, anionic and nonioinic detergents, as well as detergents in combination with alkalinized glutaraldehyde, quaternary ammonium, and phenolic disinfectants	No significant difference in the inactivation achieved by detergent type or method of virus deposition. All laundering methods achieved significant reductions of vaccinia virus however only glutaraldehyde based disinfectant in combination with detergents was able to completely inactivate vaccinia virus by >4.9 log and >4.4 log when applied by direct contact and aerosol, respectively. Poliovirus was still detected in all trials. Disinfection with detergents and gluteraldehyde resulted in 5.3 and 4.0 log reduction when applied by direct contact and aerosol, respectively. When evaluating the rinse water for the presence of virus they observed that no vaccinia could be recovered however up to 3 log pfu/ml of poliovirus could be recovered in some trials	Sidwell et al. (1970)
Cotton and wool fabrics: Wool blanket Washable wool shirting Cotton sheeting Cotton terry cloth Dull nylon jersey Dacron/Cotton Shirting	Poliovirus	Fabrics inoculated by aerosol or direct contact Washed with anionic and nonionic detergents in 44 L of 21-27, 38-43, and 54-60°C	The average reduction in viral titer of poliovirus by laundering with detergent on all surface types inoculated by direct contact and aerosol was 5 and 4.2, 3.7 and 2.5, and 3.3 and 2.04 log $CCID_{50}$/ml in hot, warm, and cold water, respectively Poliovirus could be detected in warm and cold wash water in titer of up to 3.9 log Direct contact tended to be inactivated more easily than aerosol.	Sidwell et al. (1971)

Model Protocols

The Centers for Disease Control (CDC) as well as other organizations have produced guidelines for cleaning up diarrhea/vomit associated with HuNoV outbreaks. The guidelines recommend that initial soiling needs to removed before decontamination can be successfully achieved. Certain protocols recommend that after removing initial soiling the area should be washed with hot water and some type of detergent. This step is especially important when cleaning up feces as the high organic load can inhibit subsequent decontamination. Following initial removal and cleaning of the soiling, chlorine bleach at a concentration of 5000 ppm should be applied to the area for no less than 5 minutes (Barker et al. 2004; Hall et al. 2011). After 5 minutes the area should be rinsed with water. Some protocols also recommend cleaning all areas within 25 ft. of soiling with 200 ppm bleach when outbreaks occur in a food preparation location to ensure the inactivation of viral particles that may have been generated by aerosols (The Stomach Bug Book). Carpets and other soft surfaces require special cleaning instructions, as bleach cannot be used on these surfaces. The same steps may be followed for cleaning up the initial soiling, however, decontamination requires the use of a chemical disinfectant as well as steam cleaning at 170°F for 5 min or 212°F for 1 min. While these steps are useful in containing an outbreak, additional studies need to validate the above guidelines, the actual spread radius of aerosol particles, as well as other disinfectants that can be used on surfaces where beach is not ideal for use.

Conclusion

The aim of this review was to document the role of soft surfaces in the transmission of HuNoV and identify current methods for decontamination of the virus from those surfaces. Epidemiological evidence has shown that the transmission of HuNoV is possible and most likely due to inefficient decontamination. Decontamination can be difficult on soft surfaces for many reasons, i.e. increased binding of the virus and inactivation of sanitizers. Therefore the goal of this study was to determine a successful method of decontamination that may be applied to all soft surface types. The following are the objectives of my thesis research:

- Objective 1: Optimization of recovery methods for microorganism bound to soft surfaces.
- Objective 2: Evaluate the recovery efficiency of FCV and MNV bound to glass, polyester, and cotton.
- Objective 3: Determine the efficacy of disinfectants against FCV and MNV bound to glass, polyester, and cotton.
- Objective 4: Assess the role of FCV and MNV as surrogates for HuNoV.

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CHAPTER TWO

OPTIMIZATION OF SOFT SURFACE RECOVERY METHODS USING *Escherichia coli*

Abstract

Adequate recovery methods are needed to effectively assess the role of soft surfaces in the persistence, transmission, and decontamination of pathogens. We investigated the efficiency of three elution-agitation methods for recovery of *Escherichia coli* from cotton swatches. Our results show stomaching, vortexing, and sonication were equally efficient ($P>0.05$) at recovering bacteria from cotton. The highest recovery efficiency (RE) was achieved using stomaching at 260 rpm for 5 min resulting in 30% RE. We further investigated the combined efficiency of sonication and stomaching. Using sonication for 5 min at 40 kHz prior to stomaching increased recovery efficiency to approximately 65%. Our results clearly indicate that soft surface pretreatment with sonication can enhance the bacterial recovery using stomaching.

Introduction

Soft, porous surfaces, such as textiles, are used in a wide variety of settings including the home, hospitals, schools, and offices. Textiles found in these settings include carpets, upholstery, mattresses, cleaning cloths, worker garments, and hospital

linens, such as sheets or gowns. Soft surfaces need to be evaluated for cleanliness both in terms of organic soiling and microbial burden (Hoborn and Nysrtom et al. 1985). The microbial burden is especially important as some textiles act as reservoirs for microorganisms (Tuladhar et al. 2012). Microorganisms can be transmitted from these surfaces either by direct contact or aerosolization of pathogens by foot traffic on carpets or shaking of linens (Fijan et al. 2012; Lankford et al. 2006). This has been documented as a serious threat for pathogen transmission and persistence in hospitals and long-term care facilities. Perhaps the most dangerous demonstration of the ability of soft surfaces to release bound pathogens would be the reaerosolization of *Bacillus anthracis* (Anthrax) spores from carpets during the 2001 terrorist attack on the postal service (Estill et al. 2009; Pellar et al. 2004).

In order to properly control the spread of human pathogens, it is important to understand the role of textiles in the environmental persistence, transmission, and disinfection of microorganisms (Lankford et al. 2006; Lopez et al. 2013). A crucial factor in assessing these issues is the recovery and enumeration of pathogens from textiles. The methods that have been used to recover microbes on textiles can be separated into two main categories: destructive or non-destructive elution (Rabuza et al. 2012). Destructive elution processes are those that render the textile unusable, whereas non-destructive methods leave the textile unaltered. Traditional nondestructive methods include the use of impression sampling using selective agar, scrapping onto a sterile surface, and swabbing. Destructive sampling methods include maceration, agitation, or direct agar overlays. The main disadvantage associated with some of the traditional methods, such as impression sampling, scrapping, swabbing, and direct agar overlay, is

they only recover microorganisms from the surface of the textile (Rabuza et al. 2012). This can be especially problematic for textiles as they have a more complex 3-dimensional structure than hard surfaces and may have microbes bound below the surface. In order to better assess the microbial burden on textiles, destructive sampling as well as more complex nondestructive sampling has been used. Maceration, agitation, and forced desorption have been shown to be the superior methods for textile sampling, as they increase the physical force used and the contact with the eluent (Cody et al. 1984; Fijan et al. 2013; Rabuza et al. 2012)

Of these methods, agitation has been demonstrated to be highly efficient, reproducible, and easier to perform than maceration (Cody et al. 1984). It is important to note that agitation has been considered both nondestructive and destructive. It is our opinion that agitation is nondestructive unless samples must becut. Some common methods of agitation used include shaking or rotating, vortexing, and stomaching. These methods have also been used in combination with other methods, especially sonication (Pellar et al. 2006). The aim of this study was to determine what method or combination of methods will yield the highest recovery efficiency from simple textiles using *Escherichia coli* as a biological agent.

Materials and Methods

Bacterial Culture Condition

Escherichia coli 25922 (ATCC, Manassas, VA) was used for the recovery trials. The culture was revived from stock kept at -80 **°C** by completing two passages of growth on trypticase soy agar (TSA) (Difco) at 37 **°C** overnight. A loopful of culture was inoculated

to tryptic soy broth (TSB) (Acumedia, Neogen Corporation, Lansing, MI) overnight at 37 **°C** before use.

Bacterial Recovery Trials

Recovery trials were performed on 25 x 25 mm swatches of 100% cotton (Wal-Mart). Swatches were boiled for 5 min to remove traces of finishing chemicals and autoclaved at 121 °C for 15 min. *E. coil* strain 25922 was inoculated to each swatch by spotting 200 µl of an overnight culture diluted in 0.85% saline to approximately 2 x 10^5 cfu/coupon and allowed to air dry in a laminar flow hood for 40 min. Swatches were then placed in 10 ml of phosphate buffered saline (PBS) containing 0.02% TWEEN80 (PBST) (Fisher Scientific, Hampton, NH), and several different methods for recovery were performed. These methods included sonication for 5 and 20 min at 40 kHz (Fischer Scientific FS110D), stomaching for 5 min at 260 rpm (Stomacher 400 Circulator, Seward, West Sussex, UK), and vortexing vigorously for 2 min using a standard laboratory vortex (VWR, Radnor, PA). These methods were tried individually and in combination. Each experiment, at least 3 trials were conducted.

Bacterial Enumeration

Recovery of *E. coli* from the swatches was determined by spiral-plating 50 µl serial dilutions of the recovery liquid (Autoplate 4000, Spiral Biotech, Norwood, MA). These samples were plated in duplicate on TSA and incubated for 24 h at 37 °C.

Recovery Efficiency

Recovery efficiency (RE) was expressed as the ratio of recovered bacterial population divided by the initial inoculum level.

Statistical Analysis

Statistical analysis was performed using SigmaPlot (Systat Software Inc., San Jose, CA) to perform One Way Analysis of Variance in order to determine if the RE achieved was significantly different at a level of $\alpha = 0.05$. To identify which method was most effective for bacterial recovery, the Student-Newman-Keuls Method was used to perform multiple comparisons at a significance level of $\alpha = 0.05$.

Results

Recovery of *E. coli* from Cotton Swatches

In this study, several approaches were tested to recover *E. coli* from cotton swatches, i.e., stomaching, vortexing, and sonication. For each experiment, 200 µl of *E. coli* at $1x10^6$ cfu/ml was applied resulting in approximately 2×10^5 cfu/coupon. Drying in a laminar flow hood for 40 min was sufficient for absorption of the inoculum by cotton. Stomaching for 5 min at 260 rpm, vortexing for 2 min, and sonication for 5 and 30 min at 40 kHz recovered approximately 21-30% of *E. coli* from cotton (**Figure 2.1**). Recovery using each method was not significantly different ($p>0.05$),, however, stomaching for 5 min at 260 rpm was found to be the most effective method for recovering *E. coli* from cotton swatches achieving a 30% recovery efficiency (RE). The use of sonication before and after stomaching was also evaluated as a method to enhance bacterial recovery. Using a combination of sonication for 5 minutes at 40 kHz followed by stomaching for 5 min at 260 rpm recovered significantly more ($p<0.05$) than stomaching alone or stomaching then followed by sonication. Using sonication+stomaching approach, a 65% RE was achieved (**Figure 2.2**).

Discussion

The efficiency of any given recovery method will be affected by the organism being recovered, eluent type, and surface from which it is recovered (Cody et al. 1984; Da Silva et al. 2011; Rabuza et al. 2012). This makes assessing textiles difficult because they can vary greatly in composition and construction. Determining recovery efficiency is of importance in assessing cleanliness, environmental persistence, transmission, and disinfection. Rabuza et al. (2012) stated the most common methods used for sampling microorganisms from textiles are non-destructive methods, such as RODAC contact plates and swabbing. The authors, however, note that these methods are problematic as only the microorganisms on the surface are recovered. Due to the low recovery associated with these methods alternative "destructive-elution" methods have been studied. Rabuza et al. (2012) evaluated the recovery efficiency of four sampling methods on textiles inoculated with S*taphylococcus aureus* and *Klebsiella pneumonia.* Using "traditional methods" of swabbing and contact plates, they reported a RE of 0.001%. When two destructive-elution methods (shaking and forced desorption) were used, they achieved a higher RE of 0.1%. While RE was still relatively low the destructive-elution methods outperformed the traditional non-destructive methods by 2 logs. Hoborn and Nystrom (1985) also documented similar results comparing contact plates with stomaching. Stomaching for 3 min was able to recover 10^7 cfu/cm^2 of enterococci from 12.5 cm^2 of artificially contaminated cotton that was immersed in a suspension containing an 18 h old culture. From contaminated cloth, contact plates recovered an average of 10-10^3 cfu/cm^2 of enterococci. The low recovery rates observed using traditional methods (contact plates

and swabs) are attributed to the fact they are unable to remove pathogens from the subsurface.

The results documented by Rabuza et al. (2012) and Hoborn and Nystrom et al. (1985) demonstrate that even using alternative methods, recovery of pathogens from textiles is typically very low, between 0.1 and 10%. Similarly, Coughenour et al. (2011) found that stomaching flannel cloth for 1 min resulted in a 0.1-1% RE of methicillin-resistant *S. aureus* (MRSA). Callahan et al. (2010) also reported that vortexing carpet and cotton resulted in approximately 10% RE of MRSA and vancomycin-resistant *Enteroccocus faecium* based on the log reduction reported for their disinfection studies.

Due to the low RE reported in many studies we sought to determine a method that could increase the RE of pathogens from fabrics. Three agitation methods including vortexing, sonication, and stomaching were evaluated in this study. In order to further enhance the recovery, 0.02% Tween®80 was added to PBS as the elution buffer for all experiments. Many studies have highlighted the role of the elution buffer in pathogen recovery. The addition of Tween®80 as a nonionic detergent enhances elution by disrupting the hydrophobic interaction between the bacteria and attachment surface. Tween®80 not only promotes elution but also helps to prevent microorganism binding to the surface used for recovery, such as centrifuge tubes or stomaching bags (Da Silva et al. 2011; Rose et al. 2004). Using PBS+0.02%Tween®80 in all recovery methods, we were able to recover ≥ 21% of *E. coli* from cotton. We found that stomaching for 5 min at 260 rpm resulted in the highest RE of approximately 30%. Our RE results were much higher than those that have been documented using the same methods: such as a 0.1-1% and 10% RE using stomaching and vortexing, respectively (Callahan et al. 2010; Coughenour

et al. 2011). In both studies recovery methods were applied for a short amount of time (1 min) whereas our methods were applied for a longer time (2-5 min). The extended recovery period along with the use of a surfactant may explain the higher RE found in this study. The results we obtained are in agreement with those reported by Cody et al. (1984) who investigated the RE of several sampling methods for *E. coli* and *S. aureus* from terry cloth and sheets. Using a 90 sec agitation method consisting of 590 oscillations per min in a paint shaker, the RE of *E. coli* and *S. aureus* were 57 and 31% from sheets, and 74 and 57% from terry, respectively. The lower RE reported is similar to what we found for a single recovery method (21-30%). Although Cody et al. (1984) reported a relatively short recovery period, they used a high number of oscillations per minute which may explain their higher reported values for RE.

Stomaching, vortexing, and sonication were chosen because each method increases the mechanical agitation exerted on a surface in different ways. Vortexing relies on the shearing force of the recovery liquid on the surface and is more vigorous than traditional shaking or rotating (Rose et al. 2004). Stomaching is especially useful when recovering from soft surfaces due to the combination of both shearing force and compression provided by the paddles. The shear force causes the eluent to be swept from side to side while the alternating compression drives the liquid deeper into the surface allowing pathogens that can be contained in the microenvironment, such as veins and capillaries in fabrics, to be efficiently eluted (Sharpe and Jackson. 1972). Sonication relies on physical, mechanical, and biological agitation produced by cavitation. Sonication weakens cell walls and breaks apart microbial aggregates due to pressure

buildup caused by surface resonance as well as through the generation of free radicals (Joyce et al. 2003).

In our study all three methods were capable of achieving a higher rate of recovery than those previously reported for both non-destructive and destructive methods. While the results obtained using these three methods were not significantly different, we determined stomaching to be the best method of recovery for several reasons. Stomaching yielded the highest rate of recovery of approximately 30%. In addition stomaching allows multiple samples to be processed at once with very little handling whereas vortexing can only be used on one sample at a time and must be done by hand. Sonication also allows for multiple samples to be processed,, however, prolonged use of a sonication bath will cause the temperature of water to rise, which could affect recovery. Perhaps most importantly is that stomaching provides a set amount of force based on the amount of time and rotations per minute it is applied for. The force provided by vortexing can vary based on the user and the force provided by sonication can change based on the distance and orientation of the sample with regards to the source of sonication (Puleo et al. 1967). Based on these variables, stomaching was chosen as the most efficient and reproducible method in our study.

Because of the ability of sonication to breakup microbial aggregates and biofilms, it has been used to facilitate the recovery of *Bacillus* spores, which are prone to clumping, from both porous and non-porous surfaces (Pellar et al. 2006; Rose et al. 2004). We evaluated the efficacy of using sonication before and after stomaching as a means to increase the efficiency of recovery. When using combined methods we found that it was possible to increase the RE to approximately 65% using sonication for 5 min

at 40 kHz followed by stomaching for 5 min at 260 rpm. These results are higher than those previously reported by Cody et al. (1984) who obtained a maximum of 57% RE when recovering *E. coli* from fabrics. Our results from this study were similar to those reported by Estill et al. (2009) who evaluated the RE of aerosolized *Bacillus anthracis* Sterne spores by swabbing, wiping, and vacuuming from steel and carpet. They found the highest RE from carpets was achieved by wiping resulting in a RE of 23%. Swabbing and vacuuming were found to have lower RE of 12% and 4.7%, respectively. Due to the low RE, Estill et al. (2009) assessed the carriers for residual contamination by stomaching the carpet for 4 min on high after the initial sampling. Using stomaching they were able to recover additional 64% of spores from carpets.

In our study pretreatment with sonication was found to increase the RE higher than that achieved using either stomaching or sonication alone or stomaching followed by sonication. When stomaching was done prior to sonication there was no apparent synergistic effect as the RE was the same as was reported for stomaching alone. This effect (or lack of synergism) was also documented by Bjerkan et al. (2009) who reported no difference in RE between sonication alone and scraping followed by sonication. The results reported by Bjerkan et al. (2009) as well as our own indicate that sonication as a pretreatment increases the efficacy of stomaching. This is most likely due to the ability of sonication to break up microbial aggregates. Pretreatment of the surface using sonication may weaken the bonds between groups of bacterial cells as well as between the bacteria and the surface. This in turn will make the bacteria more susceptible to the agitation provided by stomaching, as they may already be loosely adherent.

There are several factors that make performing and assessing the recovery of pathogens from textiles difficult. Due to the lack of standardization in recovery techniques, there can be a large variation in the time and speed with which recovery is conducted. In addition the efficacy of recovery can be affected by varying fiber type, fabrication, and adsorption capabilities of textiles. The ability of a bacterial cell to attach to a surface can be greatly affected by the surface topography (Verran and Whithead, 2006). "Rough" surfaces tend to promote stronger binding as they convey a greater surface area for the cell to attach to. Verran and Whitehead (2006) define roughness as irregularities in surface texture and can exist as variations in height or spacing. Surface roughness can be measured in several ways, however, it is typically done using amplitude parameters (Ra). When the value of Ra is close to size of the bacteria, there is a greater ability for cells to not only bind to but also be retained within the surface topography. This again is attributed to greater surface area of contact between the surface feature and bacteria. Due to the fact that textiles are made up of constituent fibers, there is likely a much larger variation in surface topography than found on hard non-porous surfaces and as such we can expect more variation in soft surface recovery even when using the same method.

Both Estill et al. (2009) and Cody et al. (1984) reported comparable RE using singular recovery methods whereas our study demonstrated that combined recovery method improve microbial recovery as compared with using a single method. The differences in RE could be due to differences in surface topography on different fabric types as described above. The differences in recovery of each organism on sheets and terry could be due to a favorable microenvironment of terry that promotes adhesion. For

example the fact that *S. aureus*, a coccus, were routinely recovered in higher numbers than *E. coli* could indicate that the surface topography of terry and sheeting is longitudinal which would allow for the rod shaped *E. coli* to better adhere to the surface. Additionally Estill et al. (2009) proposed that the one reason contributing to the enhanced removal of spores using stomaching could be due to the thickness of the carpet increasing the physical contact with the stomach paddles. In our study we used thin coupons of 100% cotton. As the efficacy of stomaching is related to the amount of compression that it conveys, this could explain why additional methods were needed to achieve the same RE on cotton as was found with stomaching carpets.

Conclusion

Recovery of bacteria from textiles can be influenced by many factors including the organism, eluent, and surface type. Our results clearly demonstrated the influence of different methods on the recovery of pathogens from textiles. We observed that by using sonication as a pretreatment we were able to increase the RE as compared to stomaching alone. Based on our study, we would recommend the use of sonication followed by stomaching for both efficient and reproducible results for the recovery of bacteria from soft surfaces.

Figure Legend

Figure 2.1: Individual recovery methods used to assess the efficiency of recovering of *E. coli* from inoculated cotton swatches.

Figure 2.2: Comparison of two combined recovery methods with an individual method used to assess the efficiency of recovery of *E. coli* from inoculated cotton swatches.

Figure 2.1

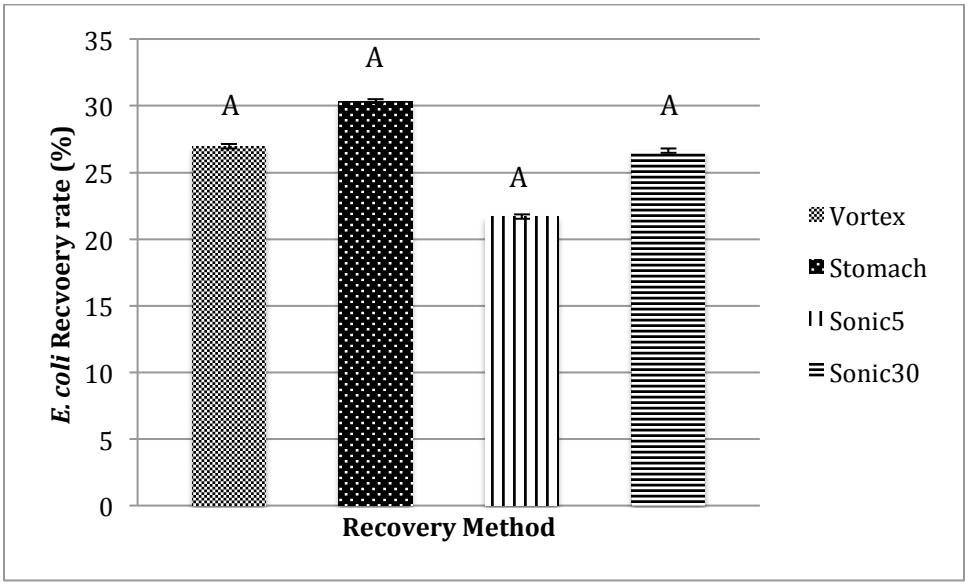
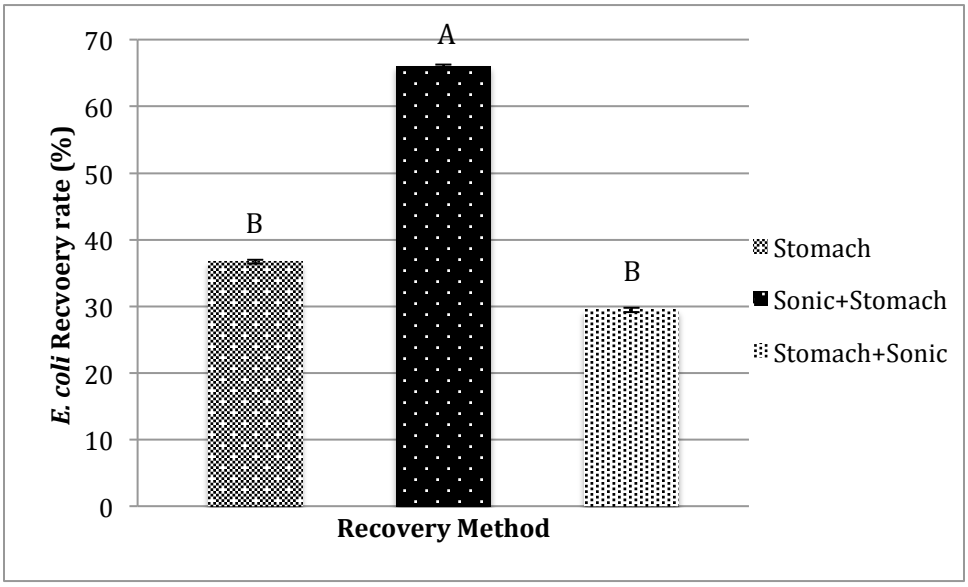


Figure 2.2



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CHAPTER THREE

RECOVERY AND DISINFECTION OF FELINE CALICIVIRUS AND MURINE NOROVIRUS FROM HARD NON-POROUS AND SOFT POROUS SURFACES

Abstract

Human Noroviruses (HuNoV) are a leading cause of foodborne disease that can be transmitted through many routes including environmental exposure to fomites. In order to control the spread of HuNoV, methods for efficiently recovering and disinfecting the virus from surfaces are urgently needed. In this study both the recovery and inactivation of two HuNoV surrogates, Feline Calicivirus (FCV) and Murine Norovirus (MNV) on glass, polyester, and cotton were evaluated by plaque assay and RT-qPCR methods. Five coupons per surface type were used to evaluate the recovery of FCV and MNV by sonication and stomaching and the disinfection of each surface using 5 ml disinfectant for a contact time of 5 min. Two sanitizers, bleach (8.25% NaOCl) and Oxivir (4.25% H_2O_2) were evaluated for disinfection efficacy. FCV at an initial titer of ca. 7 log pfu/ml was recovered from glass, cotton, and polyester at 6.2, 5.4, and 3.8 log pfu/ml, respectively, as compared with 5.5, 5.2 and 4.1 log pfu/ml, respectively for MNV with an initial titer of ca. 6 log pfu/ml. The use of bleach (5,000 ppm) was able to inactivate both FCV and MNV (2.2-4.7 log reduction) below the limit of detection on all 3 surface types. The use of Oxivir (2,656 ppm) was able to inactivate FCV (2.5-4.7 log reduction) below the limit of detection for all 3 surface types but achieved minimal inactivation of MNV (0.17-1.3 log pfu/ml). Reduction of viral RNA by bleach (5,000 ppm) corresponded to 2.72-4.06 log reduction for FCV and 2.07-3.04 log reduction for MNV on all 3 surface types.

Reduction of viral RNA by Oxivir (2,656 ppm) corresponded to 1.89-3.4 log reduction for FCV and 0.54-0.85 log reduction for MNV. Our results indicate that both virus and surface types significantly influence recovery efficiency and disinfection efficacy. Based on the performance of our proposed testing method, further improvement in virus recovery will be needed to effectively validate virus disinfection of soft surfaces.

Introduction

Human Noroviruses (HuNoV) are the leading cause of acute gastroenteritis (AGE) worldwide, responsible for >50% of cases of AGE. In the US alone HuNoV is responsible for 68% of AGE with 19-21 million cases occurring annually. While most cases of HuNoV infection are relatively mild, they cause approximately 26% of hospitalizations and 11% of deaths attributed to food borne illnesses (Hall et al. 2011; Hall, A. 2012; Kosa et al. 2013; Scallan et al. 2011).

The most common route of transmission for HuNoV is person-to-person transmission accounting for 66% of cases, 30% of which lead to secondary infections. While person-to-person transmission is the main route of exposure, there has been an increased awareness of the role of environmental transmission through exposure to contaminated surfaces and fomites that allow HuNoV to move from host to host without direct contact (Boone et al. 2007; Hall et al. 2011; Hall, A. 2012; Kosa et al. 2013; Lopman et al. 2012). This is especially relevant as the areas associated with the highest rates of HuNoV infections are settings in which persons are in close contact (Zheng et al. 2010).

The role of fomites in the spread of viral illnesses has been previously documented. Though there is little laboratory data pertaining to this route of transmission, a number of epidemiological investigations have indicated the significance of contaminated surfaces and fomites as reservoirs for viruses (Boone et al. 2007; Lopman et al. 2012). HuNoV has been detected on a variety of fomites most notably in health-care and school settings in both outbreak and non-outbreak scenarios. These surfaces can become contaminated both through direct contact with infected bodily fluids as well as indirect contact via aerosolization of pathogens from vomit and/or feces (Boone et al. 2007).

The most effective way to control the spread of HuNoV from surfaces and fomites is through disinfection. Current recommendations for disinfection of HuNoV state that between 1,000-5,000 ppm of bleach are needed to inactivate HuNoV (Barker et al. 2004, Hall et al. 2011). Both the concentration and contact time can vary based on the degree of soiling due to organic matter and the surface being cleaned (food contact vs. non-food contact). While bleach has been shown to completely inactivate HuNoV and its surrogates, the current disinfection guidelines only apply to hard non-porous surfaces. Contaminated environmental surfaces and fomites can be either hard non-porous surfaces or soft porous surfaces, the latter of which has less so been studied (Boone et al. 2007; Lopman et al. 2012).

Soft porous surfaces can be found frequently in indoor environments and can present themselves in a variety of formats including fabrics, carpets, upholstery, curtains, worker garments, personal protective equipment, and patient gowns (Boone et al. 2007; Malik et al. 2006). Disinfection of soft porous surfaces can be complicated for several

reasons. Some of these surfaces can be successfully cleaned via laundering practices, however, this method is not practical for all surface types. Also, use of bleach and other chemical disinfectants is prohibited in some settings. Finally, deleterious qualitative changes can occur during application limiting their usefullness. In addition, soft porous surfaces can exhibit a wide range of construction and composition that may have a significant influence on the attachment, survival, and subsequent transfer of viruses (Boone et al. 2007). Aside from influencing pathogen survival the soft surface matrix can also have an effect on the efficacy of chemical disinfectants (Goldsmith et al. 1954; Malik et al. 2006; McNeil et al. 1960).

Because of the inherent complexity associated with study of soft porous surfaces as well as viruses, there are currently no specific guidelines provided by either the U.S. Food and Drug Administration (FDA) or the U.S. Environmental Protection Agency (EPA) for evaluating soft surface disinfectants against viruses. The most recent guidelines for evaluating the efficacy of disinfectants for non-launderable fabrics and textiles (OSCPP 810.2400) recommend the American Society for Testing and Materials (ASTM) Test Methods for Efficacy of Sanitizers Recommended for Inanimate Non-Food Contact Surfaces (ASTM E1153-03). This method recommends the use of two fabric types, one natural and one synthetic, seeded with at least 7.5×10^5 microorganisms. In order to demonstrate successful sanitization a $\geq$ 99.9% or 3-log reduction must be achieved in the treatment as compared to the control.The EPA recommends a 4-log reduction for virucidal testing (EPA, 1981).

The aim of this study was to evaluate the impact of surface type on the recovery efficiency and develop a protocol for testing virucidal efficacy of disinfectants at inactivating HuNoV on soft surfaces, using two surrogates.

Materials and Methods

Cell Culture, Viral Propagation and Viral Stock Preparation

Crandell Reese Feline Kidney cells (CRFK) (CCL-94, ATCC, Manassas, VA) and RAW 264.7 cells (TIB-71, ATCC) were used to propagate Feline Calicivirus (FCV) strain F9 (kindly provided by Dr. Jan Vinje at CDC) and Murine Norovirus (MNV) strain CW3 (kindly provided by Dr. Virgin at University of Washington), respectively. Both cell lines were grown in a CO_2 incubator (Symphony, VWR, Radnor, PA) at 37 **°C** and 5% CO_2. CRFK cells were grown in Complete Eagles Modified Essential Media (CEMEM) consisting of EMEM (ATCC) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY) and 1% Penincillin+Streptomycin (P+S, VWR). RAW 264.7 cells were grown in Complete Dulbeccos Modified Eagle's Media (CDMEM) consisting of high glucose DMEM (Hyclone, Logan, UT) supplemented with 10% FBS, 1% P+S, 10 mM HEPES (Fisher Scientific, Hampton, NH), 1 mM non-essential amino acids (NEAA), and 2 mM L-glutamine (Hyclone). FCV and MNV were propagated by infecting respective cell lines displaying 90% confluency with 10^5 pfu/ml. Cells and viruses were then incubated at 37 **°C** at 5% CO_2 until cytopathic effects were observed under a microscope. Viral stocks of FCV and MNV were prepared from cell culture lysates. Samples showing cytopathic effect were subjected to three cycles of the

freeze-thaw method followed by low speed centrifugation at 1,200 g for 10 min at 4 °C. The supernatant was collected, aliquotted, and stored at -80 °C.

Plaque Assay

Cell culture 6-well plates (VWR) were seeded with ca. $1x10^5$ viable cells/ml of CRFK or ca. $2x10^6$ viable cells/ml of RAW 264.7 at 37 °C as described above. Once the cells reached 90% confluency (4-5 days for CRFK, 1-2 days for RAW 264.7), the cell culture media were aspirated and 500 μl of infection media were added. For FCV the infection media consisted of DMEM, 2% FBS, and 1% P+S (DMEM-2). For MNV the infection media consisted of CDMEM with 5% FBS (CDMEM-5). Following the addition of infection media 200 μl of each virus was added to each well of the 6-well plates, which were incubated for 1 h by rocking the plates every 10-15 min to make sure the inoculum was evenly spread. FCV was incubated at 37 °C and 5% CO_2, whereas MNV was incubated at room temperature. After 1 h of absorption the liquid was aspirated and a 2 ml overlay media was added. For FCV the overlay media was a 1:1 ratio of media to agarose consisting of 2X Modified Eagles Media (2X MEM) (Invitrogen) supplemented with 10% FBS and 1% P+S and 1.0 % agarose (Sigma). For MNV the overlay media was a 1:1 ratio of media to agarose consisting of 2X MEM supplemented with 10% FBS,1% P+S, 10 mM HEPES, 4 mM L-glutamine and 3% SeaPlaque Agarose (Fisher). After 48 h of incubation at 37 °C and 5% CO_2 plaques were stained for visualization. For FCV staining was achieved using a second 2 ml overlay containing 2X MEM with 0.5 % agarose and 0.6% neutral red (Carolina Biological Supply). Plaques were counted after 5-6 h incubation at 37 °C and 5% CO_2.

For MNV staining was achieved using a second 2 ml overlay containing 1% neutral red in PBS without agarose. Plaques were counted after 3 h incubation at 37 °C and 5% CO_2.

Surface Treatment and Virus Inoculation

Coupons (25 x 25 mm) of glass (VWR), polyester (100% 117acron #54) (Testfabrics, Inc.), and cotton (Testfabrics, Inc.) were used to represent a non-porous, synthetic porous, and natural porous surface, respectively. Glass coupons were prepared by dipping in 100% ethanol (Fisher), whereas cotton and polyester fabrics (ca. 300 g) were scoured through boiling for 1 h in 1 L of distilled water containing 5 g of Tergitol N-101 (Spectrum Chemical Inc.) and 5 g of Na_2CO_3 (Fisher). After boiling, fabrics were rinsed in cold tap water until no visible traces of detergent were observed. Following individual pretreatment, all coupons were autoclaved at 121 **°C** for 40 min. Coupons were inoculated by spotting 200 µl of either FCV at ca. 10^7 pfu/ml containing 5% FBS or MNV at ca. 10^6 pfu/ml (without FBS) and allowed to dry for 40 min in a humidity chamber (Thermo Scientific, Waltham, MA) at 37 **°C** with 40-42 % relative humidity (RH).

Viral Recovery Efficiency

Dried coupons were immersed in 10 ml of PBST, which consisted of PBS pH 7.4 plus 0.02% Tween®80 (Fisher) and subjected to recovery. Recovery was performed by sonication for 5 min at 40 kHz using the FS110D sonication bath (Fisher) followed by stomaching for 5 min at 260 rpm using the Stomacher 400 Circulator (Seward). The

recovery liquid was diluted in CEMEM for FCV and CDMEM-5 for MNV and 200 μl was used to determine recovery efficiency via plaque assay (**Figure 3.1**).

To assess the effect of drying on viral recovery, FCV and MNV were inoculated and recovered from 4 coupons of each surface type as previously stated using two drying times. For each surface type, 2 coupons were subjected to recovery immediately after inoculation at time 0 while the remaining 2 were recovered after drying at time 40. All samples were assessed via plaque assay. Three trials were conducted to determine the recovery efficiency of each virus.

Disinfectant Preparation

Two disinfectants were evaluated in this study. Bleach was prepared using commercially available Clorox® (The Clorox Company, Oakland, CA) with a starting concentration of 8.25% which was diluted with sterile distilled water to 5.0% before receiving a final 1:10 dilution to achieve 5,000 ppm. The concentration of bleach was determined using AquaCheck Pool & Spa Test Strips (ElkhArt, IN). Oxivir was prepared using a commercially available solution Oxivir® Five 16 Concentrate (Johnson Diversey, Amsterdam, The Netherlands) following the instructions provided on the label. The stock solution containing 4.25% H_2O_2 was used to prepare a 1:16 working solution using sterile distilled water resulting in an approximate concentration of 2,656 ppm. All disinfectant working solutions were prepared immediately prior to disinfection tests.

Quantitative Suspension Test

A 100 µl virus stock solution of FCV (w/FBS) and MNV (w/o FBS) was mixed well with 900 μ l of the prepared disinfectant solution for a contact time of 5 min. A control was performed by mixing 100 μ l virus with 900 μ l of CEMEM for FCV and CDMEM-5 for MNV. Following disinfection 100 μ l of this mixture was transferred to 900 μ l of PBS with 10% FBS for neutralization for 5 min. The neutralized virus-disinfectant was then serially diluted in CEMEM for FCV and CDMEM-5 for MNV and assayed via plaque assay.

In order to evaluate the effectiveness of PBS+10% FBS as a neutralizer to stop the action of the disinfectant as well as to prevent cytotoxicity to the cell culture system 900 µl of the neutralizer solution was mixed with 90 μ l of disinfectant for a contact time of 5 min. This solution was then serially diluted in either CEMEM for FCV or DMEM-5 for MNV and then inoculated with 10 μ l of low titer virus (10^5 pfu/ml for FCV and 10^4 pfu/ml for MNV). Dilutions of 10^0 and 10^{-1} were then assayed via plaque assay. A total of 3 trials were conducted for each surrogate.

Surface Disinfection Test

Fifteen coupons per surface type were inoculated with HuNoV surrogates FCV and MNV and dried as previously stated. Two additional coupons served as the neutralizer/cytoxicity (N/C) control for each disinfectant and received equal amounts of CEMEM for FCV trials and CDMEM-5 for MNV trials instead of viral inoculum. This resulted in a total of 17 coupons including 5 control (C), 5 treatment #1 (T1), 5 treatment #2 (T2), and 2 N/C control (**Figure 3.2**). For treatment groups 5.0 ml of 5,000 ppm chlorine solution was added to each of 5 coupons designated as the T1 (n=5) as well as

the N/C control (n=1) and 5.0 ml of 2,656 ppm Oxivir was applied to each of another 5 coupons designated as T2 (n=5) as well as the final N/C control (n=1). The remaining inoculated coupons (n=5) received 5.0 ml of CEMEM or CDMEM-5 to serve as a positive control. After 5 min of contact time all samples were neutralized for 5 min in 10 ml of PBST+10% FBS. Following neutralization virus was recovered by sonication and stomaching as previously stated. Eluent from the treatment and control samples received 10-fold dilution in CEMEM or CDMEM-5. After recovery the solution from the N/C coupons was mixed with low titer virus (10^2 pfu/ml for FCV and MNV) and allowed 5 min contact time to simulate disinfection conditions. All samples were assayed via plaque assay as well as through RT-qPCR as described below. A total of 3 trials were conducted for each surrogate.

Surface Disinfection Using Different Concentrations of Bleach

Nine coupons of cotton were inoculated with FCV as previously stated to serve as a control (n=2), treatment #1 (T1) (n=2), treatment #2 (T2) (n=2), treatment #3 (T3) (n=2) and N/C control (n=1) to test the efficacy of 5,000 (T1), 500 (T2), and 50 (T3) ppm bleach. The N/C control was performed using the highest concentration of bleach used (5,000 ppm). Surface test were performed as previously described and assessed via plaque assay. Two trials were performed.

RNA Extraction and RT-qPCR

Viral RNA was extracted from 140 µl of recovery liquid or virus stock using the QIAamp Viral RNA MiniKit (QIAGEN). Extracted RNA was stored at –80 °C prior to

use. RT-qPCR was performed using the KAPA SYBR Fast Universal One-Step RT-qPCR Kit (KAPA Biosystems). The PCR reaction was carried out on a Realplex2 Mastercycler (Eppendorf). Primers for the RT-qPCR reaction were obtained from Invitrogen using sequences as described by as Park et al (2010). The forward and reverse primer sequence for FCV was GCCATTCAGCATGTGGTAGTAACC and GCACATCATATGCGGCTCTG, respectively. The forward and reverse primer sequence for MNV was TGATCGTGCCAGCATCGA and GTTGGGAGGGTCTCTGAGCAT, respectively.

Optimization of RT-qPCR was done by performing standard curve analysis for each virus. The standard curve for FCV and MNV were prepared by performing a 7 step 10-fold dilution of FCV at a starting concentration of 2×10^7 pfu/ml and using a 7 step 5-fold dilution for MNV at a starting concentration of 6.65×10^5 pfu/ml. The parameters for PCR the cycle consisted of 5 min at 42 °C for cDNA synthesis followed by 5 min at 95 °C to inactivate the reverse transcriptase. The DNA amplification was conducted with 40 cycles of denaturation at 95 °C for 3 sec followed by annealing at 60 °C for 20 sec, and to confirm the positive amplification a final step of 72 °C for 20 sec was used to allow for complete data acquisition. Melting curve analysis was performed at 95 °C for 15 sec, 60 °C for 15 sec, 20 min data acquisition period, and finally 95 °C for 15 sec.

Log reduction of RNA for RT-qPCR was calculated by $(Ct_t-Ct_c)/k$ where Ct_t is the cycle threshold for treatment group, Ct_c is the cycle threshold for the control group, and k is the slope obtained from plotting the Ct values versus the log10 of the RNA copy number used for presenting the standard curve (Park et al. 2010).

Statistical Analysis

Statistical analysis was performed using SigmaPlot software (Systat Software Inc., San Jose, CA). Kruskal-Wallis One Way ANOVA on ranks was performed at a significance level of $\alpha \leq 0.001$ to determine differences in recovery from each surface type for FCV and MNV. Pairwise multiple comparison using $\alpha \leq 0.05$ was performed by Dunn's Method for FCV and the Student-Newman-Keuls method for MNV. Differences in recovery between FCV and MNV from the same surface type was determined using the Mann-Whitney Rank Sum Test at a significance level of $\alpha < 0.001$. To determine the effect of drying on the recovery of FCV and MNV a t-test was used at a significance level of $\alpha < 0.05$. The Mann-Whitney Ran Sum test was performed using a significance of $\alpha < 0.001$ when the normality or equal variance test failed.

Results

Virus Recovery Efficiency

Recovery efficiency (RE) was determined based on the average of recoverable virus titer from each surface type compared with the initial titer. The average of recoverable viral titer for FCV from glass, polyester, and cotton was 6.2, 5.4, and 3.8 log pfu/ml, respectively (**Figure 3.3)**. Recovery of MNV resulted in an average of 5.5, 5.2, and 4.1 log pfu/ml from glass, polyester, and cotton, respectively (**Figure 3.3**). The best RE was obtained from glass for both FCV and MNV resulting in a 35.22 and 24.27% RE, respectively. Polyester and cotton exhibited a lower RE of 5.59 and 0.15% for FCV and 14.69 and 0.85% for MNV, respectively. Recovery was significantly different ($P < 0.05$) across allhree surface types,, however, the REs of FCV and MNV were only significantly different from cotton ($P < 0.001$) (**Table 3.1**).

Assessment of Drying Time on Viral Recovery

The effect of drying for 40 min on viral recovery was significant for both FCV ($P<0.05$) and MNV ($P<0.001$) on polyester and cotton but not on glass (**Figure 3.4**). Recovery of FCV and MNV at time 0 from all three surface types ranged from approximately 6.4-6.5 log pfu/ml and 5.5-5.7 log pfu/ml, respectively. After drying for 40 min, significant differences in recovery of FCV and MNV from polyester and cotton were observed resulting in 1.46 and 3.04 log pfu/ml reduction for FCV and 0.53 and 1.44 log pfu/ml reduction for MNV, respectively, as compared with time 0.

Quantitative Suspension Test

The efficacy of disinfection was determined by calculating the difference in recoverable virus between the control and treatment groups. Both FCV and MNV were reduced below the limit of detection by bleach (5,000 ppm) and Oxivir (2,656 ppm)**.** As there was no recoverable virus from the treatment group, log reduction was calculated by subtracting the limit of detection (1.39 log pfu/ml) for the plaque assay from the control values to allow for a more accurate assessment of disinfection. This resulted in approximately 5.5 log reduction for FCV and 4.3 log reduction for MNV (**Table 3.2**).

The PBS+10% FBS solution was determined to be successful at neutralizing both sanitizers, however, an additional 1:10 dilution was needed to prevent cytotoxicity caused by both Oxivir and bleach. Using a 10^{-1} dilution of the N/C control, 10^{2} pfu/ml of FCV and MNV could be detected by plaque assay, with no difference ($p>0.05$) in viral titer as compared to the initial inoculum.

Surface Disinfection Test

Plaque Assay. FCV was reduced below the limit of detection (1.39 log pfu/ml) by plaque assay using both bleach and Oxivir on all 3 surface types. This resulted in a reduction of approximately 4.7, 4.1 and 2.5 log pfu/ml of FCV on glass, polyester, and cotton (**Table 3.3**). MNV was reduced below the limit of detection (1.39 log pfu/ml) on all 3 surface types using bleach,, however, less inactivation was observed using Oxivir. Reduction of viral titer of MNV by bleach resulted in approximately 3.8, 3.6, and 2.2 log pfu/ml as compared with 1.3. 0.57, and 0.17 log pfu/ml by Oxivir on glass, polyester and cotton, respectively (**Table 3.3**). The use of PBST+10% FBS was successful at neutralizing the virucidal and cytotoxic activity of both disinfectants at the 10^0 dilution.

RT-qPCR. Analysis of the standard curve obtained by performing a 7 step 10-fold dilution of FCV at a starting concentration of 2 x 10^7 pfu/ml provided an average slope of -3.1365 with an R^2 of 0.9965 and efficiency of 1.08 (**Fig. 3.5a)**. The standard curved obtained using a 7 step 5-fold dilution for MNV at a starting concentration of 6.65 x 10^5 pfu/ml provide an average slope of -3.46 with an R^2 of 0.9995 and efficiency of 0.945 (**Fig. 3.6a)**. Melting curve analysis for FCV and MNV showed the same melting temperature for products extracted from experimental samples as those used for the standard curve. Additionally the amplification plot and melting curve for FCV (**Fig. 3.5b-c**) and MNV (**Fig. 3.6b-c**) demonstrated that C_t values obtained in the negative template control were not caused by formation of DNA and were >8 C_t values below those obtained for the lowest dilution on the standard curve (**Figure 3.5a and 3.6a**).

The reduction observed in viral RNA of FCV corresponded to 4.06, 3.73, and 2.72 log pfu/ml by bleach and 3.40, 3.36, and 1.89 log pfu/ml by Oxivir on glass, polyester and cotton, respectively. The reduction in viral RNA of MNV was approximately 2.20, 3.04, and 2.72 log pfu/ml using bleach and 0.85, 0.85, and 0.54 log pfu/ml using Oxivir on glass, polyester and cotton, respectively (**Table 3.4**). FCV appeared to be more susceptible to bleach (5,000 ppm) on glass than MNV demonstrating a 1.89 log pfu/ml higher reduction in viral RNA,, however, the initial titer for MNV was lower than FCV. The reduction in viral RNA measured by RT-qPCR differed from the observed reduction in viral titer by plaque assay for FCV and MNV by approximately 0.61-1.3 and 0.13-1.6 log pfu/ml, respectively.

Surface disinfection test using various concentrations of bleach

FCV was reduced below the limit of detection (1.39 pfu/ml) on cotton using 5,000 and 500 ppm bleach achieving a reduction of 2.5 log pfu/ml in viral titer. When 50 ppm bleach was used no reduction in viral titer was observed (**Table 3.5)**.

Discussion

To control the spread of HuNoV in the environment one must understand the role of transmission due to fomites. It is necessary to properly assess the amount of HuNoV on a surface as well as the ability to decontaminate a surface of HuNoV. Part of our procedure involved a combined method for enhancing the recovery of pathogens from soft porous surfaces, which in the past has shown low recovery rates (Cody et al. 1984; Puleo et al. 1967; Rabuza et al. 2012). The proposed protocol was used to evaluate the

role of surface and virus type on the efficiency of recovery as well as efficacy of decontamination using commercial disinfectants.

Our results indicated that surface and virus type had a significant influence on the recovery efficiency. We found that both FCV and MNV exhibited higher recovery efficiency when inoculated onto glass than either polyester or cotton. In addition the recovery of both viruses from cotton was drastically lower than that of polyester. MNV exhibited a higher recovery from soft porous surfaces, however, it was only significant for cotton. The results we obtained are most similar to those reported by Gibson et al. (2012) who evaluated the removal and transfer of viruses from non-porous and porous surfaces. They found that FCV and MNV could be recovered from non-porous surfaces at an efficiency of 41 and 57%, respectively, as compared with an average 36 % recovery of FCV from four different cleaning cloths. Due to issues with the plaque assay, the results for transfer of MNV were not reported. Another study on the transfer of pathogens from porous and non-porous surfaces (Lopez et al. 2013) also showed recovery efficiencies similar to the range we observed. These investigators found that MS2, a bacteriophage, could be transferred from glass, polyester, and cotton with an efficiency of 67, 2.3, and 0.3%, respectively.

There were several factors that may have contributed to the higher reported RE in other studies as compared to ours, most notable differences were drying time and temperature, length of recovery, and more modified eluents. In the studies done by Gibson et al. (2012) and Lopez et al. (2013) the virus on the surfaces was allowed to dry at room temperature (19-25 **°C**), whereas our inoculated surfaces were dried at 37 **°C**. As FCV and MNV have been documented as being most stable at 4 **°C**, with decreasing

stability at room temperature (approximately 25 °C) and 37 °C, there may have been a greater initial loss due to drying in our study (Bae and Shwab. 2008; Cannon et al. 2006; Doultree et al. 1999; Kim et al. 2012). We chose 37 °C for drying time as it is recommended in the ASTM method E1153-03 for evaluating surface sanitizers. Additionally, the methods reported by Lopez et al. (2013) used a recovery time of 30 min with a buffer modified to include salts, surfactants, and amino acids. In our protocol we used a total recovery time of 10 min with an eluent only modified to include a surfactant. An increased recovery period may have allowed for virus bound to the subsurface to be better eluted and the addition of the modified eluent may have allowed for a greater disruption in the virus and the surface binding interaction. Though the reported recovery efficiencies are different than our own, we can see that pathogens are consistently being recovered more efficiently from non-porous surfaces than from porous surfaces. Recovery of viruses from porous surface has historically been lower than that of non-porous surfaces, which has been attributed to a greater ability of viruses to become attached and trapped within the fibers of the soft surfaces. While this is a significant factor, the differences in recovery due to surface type is likely influenced by several factors.

The hydrophobicity of a surface type is one factor that plays a large role in the recovery of viruses from porous surfaces. Polyester is hydrophobic, whereas cotton is hydrophilic (Lameiras et al. 2008; Zuo et al. 2013). Glass is also considered hydrophilic,, however, the influence that the surface has on adsorption is more affected by its porosity (Thompson et al. 1998). As a hydrophilic surface, cotton exhibits rapid adsorption of the inoculum allowing viral particles to be completely dispersed across the surface and

providing a greater surface area for viral attachment. The hydrophobic nature of polyester, however, promotes droplet formation on the surface which results in less overall surface area for the virus to become attached. Although polyester also allows the inoculum to be absorbed, we observed that the rate of absorption and subsequent dispersion across the surface was much slower than that of cotton. On glass surfaces the inoculum was easily spread over the entire coupon allowing for sufficient surface area for attachment though as a non-porous surface the virus containing media was unable to become saturated within the subsurface. The effect of hydrophobicity on attachment was demonstrated by Zuo et al. (2013) in evaluating the recovery efficiency of avian influenza virus (AIV) dried onto three soft porous surfaces, 2 of which were hydrophobic in nature and one that was hydrophilic. They reported between 40-50% RE of AIV from hydrophobic surfaces (polypropylene and polyester) and 30% RE from the hydrophilic surface (nylon) after drying. These authors observed the promotion of droplet formation on hydrophobic surfaces versus full saturation seen on the hydrophilic surface and suggest that their low recovery efficiency from nylon is due to the faster absorption of the virus containing medium bringing the virus closer to the surface and allowing more contact for binding. Sattar et al. (2001) also documented an increased ability of bacterial cells to be transferred from gowns that were made out of a polyester-cotton blend than those made of cotton alone. This difference was attributed to the presence of polyester increasing the hydrophobicity of the fibers thereby reducing the ability of the bacterial cells to bind deep within the fibers.

Zuo et al. (2013) and Sattar et al. (2001) focused on the effect of hydrophobicity on attachment due to absorption, however, the rate of absorption is also going to

influence the resistance of a virus to desiccation. The rapid absorption of liquid by hydrophilic surfaces results in a more rapid evaporation of the virus containing medium. This results in much faster drying time than seen on hydrophobic surfaces and direct exposure of the virus to the effects of temperature and ambient environment without the protection of moisture from the medium. This effect could be another reasons why glass, which takes the longest time to dry, exhibited the greatest recovery efficiency. Zuo et al. (2013) discussed the role of desiccation on recovery as they observed that recovery decreased as drying time increased. As the observed differences among surface types were evident at time 0, in which there would be little due to desiccation, they indicated that virus binding interaction was the most significant influence due to surface type. In our study, however, we observed no difference ($p>0.05$) in recovery due to surface type before drying. This indicates that the influence of hydrophobicity of the surface is more significant due to its effect on drying time rather than attachment alone. This is also supported by Hall et al. (1980) who evaluated the recovery of respiratory syncytial virus (RSV) on non-porous and porous surfaces. They determined that RSV could be detected for up to 7 h on countertops, 2 h on cloth gowns, and only 30 min on paper due to survival of the pathogen. In addition when drying RSV onto glass they found that spreading the inoculum over a larger surface area, to facilitate a faster dry time, resulted in 10-log less recovery in viral titer than when allowed to dry normally. In our study, the effect of drying may also explain why FCV exhibited a higher RE than MNV for glass alone. Drying at 37 **°C** may result in more rapid inactivation of MNV than FCV resulting lower recovery. As reported previously, FCV has been reported as being more stable than

MNV at 56 **°C** and capable of persisting for longer under dry conditions at 22 **°C** (Cannon et al. 2006; Cromeans et al. 2013).

Another factor influencing the virus recovery due to surface and virus type is the notable differences in reported isoelectric points. The isoelectric point (IEP) is defined as the pH at which the net charge of a virus or surface is zero. At pH above the IEP the net charge will be negative whereas below the IEP the net charge will be positive. When both the virus and the surface exhibit the same charge, the electrostatic repulsion between the two will be the greatest and will facilitate removal of the virus from the surface (Gerba, C.P. 1984). The reported IEP for glass, polyester, and cotton are 2.1, 2.3, and 2.8-3.0 (Bellman et al. 2005; Lameiras et al. 2008). In general it can be said that the higher the IEP of a surface the more likely it is to promote attachment (Gerba, C.P. 1984). This would further explain why the RE for our 3 surface types was highest for glass, followed by polyester and then cotton. This effect was also demonstrated by Zuo et al. (2013) who indicated that the IEP of nylon (5.2-6.9), which exhibited the lowest recovery, was higher than that of polyester (2.3-2.5) and polypropylene (2.9-3.8).

The role of virus IEP likely also played a role in the recovery of FCV and MNV. Dowd et al. (1998) evaluated the effect of IEP on adsorption of 5 bacteriophages to columns and found a negative correlation between IEP and virus adsorption documenting that as IEP increased adsorption decreased. The IEP of FCV has been documented as 4.9 and the IEP of MNV is proposed to be between 5.0-6.0, similar to that of HuNoV (Gibson et al. 2012; Michen & Graule. 2009). Using our recovery eluent with a pH of 7.4 both the virus and the surface would have exhibited a net negative charge, which should aid in the recovery due to electrostatic repulsion. Although both viruses should have a net

negative charge at pH 7.4, the effect on electrostatic repulsion may not be the same for both viruses. A study done by Vega et al. (2005) on attachment of FCV to butterhead lettuce demonstrated that adsorption of FCV to the surface became stronger above its IEP (pH 5.0-7.0) although no IEP was given for lettuce. Another study by Schaldach et al. (2006) on HuNoV suggests at the pH range 4-7 HuNoV becomes increasingly negatively charged and exhibited greater electrostatic repulsion from a negatively charged surface than MS2, which has an IEP of 3.9. If the assumptions regarding the correlation between IEP and adsorption and the similarities between MNV and HuNoV hold true, then this could explain why MNV was recovered more efficiently from soft surfaces despite the lower reported recovery from glass.

In addition to evaluating the role of surface and virus type on the efficiency of virus recovery, we also sought to evaluate the effect of both factors on the efficacy of disinfection. Bleach and Oxivir were chosen in this study as both are registered as disinfectants against HuNoV (EPA. 2009). Both bleach and Oxivir are oxidizing compounds whose active ingredients are sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2), respectively. The mode of action causing virus inactivation using NaOCl is not clearly understood and may be due to either destruction of the viral RNA, capsid proteins, or both. The antiviral action of H_2O_2 is attributed to the generation of free radicles ($^{\cdot}OH$), which primarily target capsid proteins, with damage to the viral RNA being a secondary mechanism of inactivation (Koivunen and Heinonen-Tanski. 2005; McDonnell and Russell. 1999).

In the present study we found that both FCV and MNV could be reduced below the limit of detection in suspension and on all 3 surface types using 5,000 ppm bleach.

We also assessed the efficacy of various concentrations of bleach against FCV on cotton to determine if there is a dose response. We observed that 5,000 ppm and 500 ppm bleach were both capable of reducing FCV below the limit of detection, however, no inactivation was achieved using 50 ppm bleach. This effect may have been caused by some interaction due to the FCV being attached to cotton as Tung et al. (2013) reported that FCV could be reduced by approximately 1 log using 75 ppm bleach in suspension. Though the use of bleach has not been evaluated on soft surfaces outside of laundering practices, its effectiveness on HuNoV and its surrogates on hard surfaces and in laundering practices has been well documented. The concentration of sodium hypochlorite needed for virus inactivation can vary depending on several factors including contact time, organic soiling, and virus type. Tung et al. (2103) demonstrated that a 30 s contact time in suspension was sufficient to inactivate FCV and MNV by > 3 logs using 250-500 ppm bleach, however, HuNoV needed >500 ppm to achieve the same inactivation. Park and Sobsey (2011) observed that 5,000 ppm bleach was needed to achieve a 3 log inactivation of FCV and MNV after 1.9 and 3.2 min, respectively, when dried onto stainless steel using 10% fecal soiling. HuNoV, however, only showed a 1.4 log reduction after 4 min under the same conditions. Barker et al. (2004) demonstrated that HuNoV contained in fecal matter could still be detected on up to 28 % of surfaces after cleaning with 5,000 ppm bleach. HuNoV could only be completely eliminated when a precleaning step using 4% anionic detergent was used to remove soiling followed by the application of bleach. In our study there was no apparent effect of virus type, surface type, or presence/absence of soil (5% FBS) on the efficacy of bleach. The differences we reported in reduction of viral titer were due to recovery alone.

The use of Oxivir was also evaluated for its effectiveness at inactivating FCV and MNV in suspension as well as on glass, polyester, and cotton. Oxivir is an accelerated hydrogen peroxide (AHP) disinfectant containing H_2O_2 as the active ingredient along with other components such as surfactants and wetting agents. H_2O_2 is considered ideal for surface disinfection because it is generally effective against a wide range of microorganisms and breaks down to non-toxic byproducts as water and oxygen. The working solution used in this study contains approximately 2,656 ppm H_2O_2 that was prepared in a 1:16 dilution from a concentrated stock. FCV was completely inactivated below the limit of detection in suspension as well as on all 3 surface types after 5 min of contact time,, however, MNV showed resistance to the disinfectant on the surface with a decreasing efficacy in the following order: glass>polyester>cotton.

Previously reported studies on the efficacy of AHP and other H_2O_2 based disinfectants against non-enveloped viruses support the results that we obtained. Howie et al. (2008) investigated the efficacy of disinfection using 3 concentrations of AHP against several environmental pathogens including the non-enveloped Reovirus. When evaluating AHP at 70,000 ppm, 5,000 ppm, and 500 ppm, they found that only 500 ppm AHP was ineffective at inactivating pathogens on surfaces. Approximately 4 log reduction of Reovirus was achieved after 4 min and 8-10 min using 70,000 and 5,000 ppm, respectively. Sattar et al. (2004) also evaluated the effectiveness of an AHP against FCV dried onto a carrier in the presence of soiling. They observed FCV was inactivated by >4.7 logs after 3 min using an AHP containing 5,000 ppm H_2O_2. Although the concentration used in both studies was much higher than that of our study the reported inactivation rates are similar to those that we obtained on glass for FCV. The efficacy of

H_2O_2 against FCV at lower concentrations may be due to the synergistic effect of the AHP. Though surfactants are generally considered ineffective at inactivating non-enveloped viruses such as FCV, they may cause minor damage to the capsid causing the virus to be more susceptible to damage from by H_2O_2.

The incomplete inactivation of MNV in the surface disinfection tests could be due to a number of factors. Although MNV was inactivated in suspension tests, this method has been reported to have the potential to overestimate the efficacy of disinfectants. Carrier tests provide a more realistic assessment due to the interaction of the virus and the disinfectant with the surface (Abad et al. 1997; Druce et al. 1995; Malik et al. 2006). Inactivation of MNV using H_2O_2 has been achieved using a concentration or contact time significantly higher than that used in our study. Li et al (2011) reported that 21,000 ppm of H_2O_2 with a contact time of 10 min was needed to achieve a >3 log reduction of MNV dried onto stainless steel. When shorter contact time was used (5 min), <3 log reduction was achieved using the same concentration. Vaporous H_2O_2 (vH_2O_2) was found to be significantly less effective achieving a <1 log reduction on stainless steel after 5 min contact time. In our study a 5 min contact time was used in all experiments and the concentration of our solution was nearly 1:10 as dilute than that in the Li et al (2011) study. Tuladhar et al. (2012) demonstrated that vH_2O_2 can be effective at achieving significant reduction of MNV however, when extended contact time was used. When dried onto stainless steel and wooden framing panels, MNV was capable of being inactivated by >4 log reduction after 1 h contact time using 127 ppm vH_2O_2. The results of the above studies indicate that the synergistic properties of Oxivir are not enough to overcome the resistance of MNV at the concentrations recommended for use.

In order to evaluate the efficacy of disinfection we assessed our samples by plaque assay as well as RT-qPCR. The observed differences in reduction between viral RNA and infectious viral titer may be related to the mechanism of inactivation. Inactivation of viral particles can occur through damage to the viral capsid as well as the RNA and may be a factor of either one or both effects. Because of this there may be RNA present in samples where the infectivity of the virus has been completely diminished. The plaque assay method is crucial in evaluating disinfectants as it is able to determine whether viruses remain infectious after treatment. We observed that the reduction in viral RNA by RT-qPCR slightly underestimated the efficacy of disinfectants against FCV by 0.61-1.3 log pfu/ml as measured by plaque assay depending on the treatment and surface type. When evaluating MNV, we observed that RT-qPCR slightly underestimated the efficacy of bleach on all 3 surfaces by approximately 0.13-1.6 log pfu/ml and Oxivir on glass by 0.52 log pfu/ml. On the contrary, we found that RT-qPCR overestimated the efficacy of Oxivir on polyester and cotton by 0.28 and 0.37 log pfu/ml, respectively. Tung et al. (2013) reported similar findings when assessing the efficacy of bleach against MNV and FCV in suspension. When using 250 ppm bleach they observed that the infectious titer of MNV and FCV could be reduced by 3.9 and 3 logs whereas reduction in viral RNA only resulted in 3.0 and 2.5 logs respectively. Park et al. (2010) also observed significant differences in the reduction of viral RNA and infectious titers of FCV and MNV treated with ethanol. Ethanol at a concentration of 67-72% achieved a 2.6-3.6 log reduction in the viral titer of FCV and MNV as determined by plaque assay but demonstrated a 0.0-0.9 log reduction in viral RNA.

As HuNoV cannot yet be cultured *in vitro* RT-qPCR methods must be used in order to determine efficacy based on reduction in viral RNA, however, this does not allow for the infectivity of the virus particle to be determined. Because of this, RT-qPCR methods may either underestimate or overestimate the efficacy of a disinfection method. This is the major disadvantage of the RT-qPCR method and the reason why surrogate viruses should be used in order to validate adequate disinfection.

The results that we obtained pertaining to both the recovery and disinfection of FCV and MNV highlight two important factors associated with the study of disinfection of soft surfaces. Inadequate disinfection has been reported as a contributing factor in outbreaks of HuNoV, especially those related to soft surfaces. Outbreaks where soft surfaces have been implicated documented using vacuuming and shampooing to clean contaminated areas, both of which have proven ineffective. There is currently no approved method for validating disinfectants or sanitizers on non-launderable soft surfaces. This is likely due to the inherent complexity associated with the study of soft surfaces.

The main difficulty associated with soft surfaces is the low recovery of pathogens. This can make it increasingly difficult to document the adequate level of reduction needed to make disinfectant claims. This was especially apparent in the results we observed for FCV and MNV. In order to establish a disinfectant as an effective virucidal agent a 4-log reduction in viral titer is needed (EPA, 1981). Though both bleach and Oxivir were able to reduce the titer of FCV below the limit of detection on all 3 surface types an antiviral claim would only be possible on glass and polyester. The low recovery observed on cotton makes it impossible to say that the virus has been

completely inactivated. Due to this it will be especially important to further evaluate more methods of recovery in moving forward with developing a standardized method for identifying soft surface disinfectants. Though our approach of a combined recovery method was more successful than previously used methods it is clear that a more efficient recovery method is needed.

Another effect due to surface type that is less understood is the ability of a soft surface to influence the efficacy of disinfection. Oxivir was proven to be ineffective against MNV on all surface types, however, the observed inactivation was higher on glass with decreasing efficacy on either polyester or cotton. We believe that there may have been some interaction between the disinfectant and the fibers of the soft surfaces. Porous surfaces may protect viruses from disinfection due to their ability to become sequestered into the subsurface matrix, or decrease the efficacy of the disinfectant due to interaction with the fiber type. Fibers such as cotton and wool have demonstrated the ability to decrease the efficacy of quaternary ammonium compounds and sodium hypochlorite, respectively, by removing the active ingredient from solution due to an interaction with naturally occurring fiber components (Goldsmith et al. 1954; McNeil et al. 1960). In order to achieve successful inactivation disinfectant solutions must be applied at high enough concentration to overcome this effect. Specific interactions between other disinfectants and surface types have not been as clearly defined, however, the decreased efficacy on soft surfaces we observed has been observed in other studies. Li et al. (2011) documented a <1.5 log reduction of MNV on lettuce using 25,200 ppm H_2O_2 after 5 min as compared to a >2.5 log reduction using 21,000 ppm on stainless steel for the same contact time. Tuladhar et al. (2012) using vH_2O_2 for disinfection of MNV

reported a reduction of viral titer of >3 logs on gauze versus >4.5 logs on stainless steel and wooden framing panels. Reduction of RNA was lower showing a 2 and 0.5 log reduction for MNV and HuNoV on stainless steel, respectively, and no significant reduction on framing panels and gauze. Malik et al. (2006) also observed that the efficacy of disinfection could be influenced by surface type among soft surfaces. When evaluating the inactivation of FCV on fabrics and carpets, they found that out of 3 fabric and 4 carpet types tested, polyester proved to be the least susceptible to disinfection. In addition they observed that when disinfectants were applied to carpets for 1, 5, and 10 min, an increase in contact time correlated with a decrease in efficacy in 3 out of 5 disinfectants. They attributed this to the possibility of the carpet materials inactivating the disinfectants when they were allowed for longer contact. As the types of soft surfaces present in the environment can be very diverse in the construction and composition a more in depth study may be needed to fully determine a method of disinfection that is applicable across many surface types.

The difference in inactivation using Oxivir against FCV and MNV also highlights the growing concern with using FCV as a surrogate for HuNoV. The surrogate FCV is currently the EPA approved surrogate for making disinfectant claims against HuNoV. FCV has been demonstrated as being more susceptible than either MNV or HuNoV to pH and temperature as well as to organic solvents and certain disinfectants (Cannon et al. 2006; Cromeans et al. 2013, Girard et al. 2010; Poschetto et al. 2007). Both viruses were inactivated by bleach in our study but there were significant differences when using Oxivir. Though it was demonstrated to be effective against FCV in both suspension and surface tests the resistance of MNV to disinfection on surfaces could indicate that

HuNoV may not be successfully inactivated using this product. This poses a significant threat to the environmental disinfection of HuNoV. As this is a major method for the controlling the spread of HuNoV, a more suitable surrogate may be needed to identify successful disinfection practices.

Conclusions

We showed that viral recovery differed significantly from non-porous, synthetic porous, and natural porous surfaces. Additionally we determined that bleach (5,000 ppm) and Oxivir (2,656 ppm) were capable of inactivating FCV on non-porous and porous surfaces,, however, only bleach was effective against MNV. Due to the low recovery of viruses from cotton we were unable to document the 4 log reduction necessary to establish antiviral efficacy. Though the recovery efficiency we achieved was lower than expected our method was able to determine significant effects of surface type on both recovery and disinfection. Further study of efficient viral recovery methods from soft surfaces will be needed in order to develop a standardized method for the disinfection of soft porous surfaces.

Figure Legend

Figure 3.1: Flow chart for performing recovery efficiency trials.

Figure 3.2: Flow chart for performing disinfectant efficacy trials.

Figure 3.3: Recovery of viral titer for FCV and MNV obtained after inoculation of glass, polyester, and cotton surfaces allowed to dry for 40 min. Gray bars represent FCV and black bars represent MNV.

Figure 3.4: Recovery of viral titer for FCV and MNV obtained after inoculation of glass, polyester, and cotton surfaces allowed to dry for 0 and 40 min. Grey bars represent cotton, black bars represent polyester, and stripped bars represent cotton.

Figure 3.5a-c: RT-qPCR analysis of FCV recovered from control and treatment surfaces after disinfection. Figures include standard curve (3.5 a), amplification plot (3.5b), and melting curve analysis (3.5c) of two separate runs.

Figure 3.6a-c: RT-qPCR analysis of MNV recovered from control and treatment surfaces after disinfection. Figures include standard curve (3.6a), amplification plot (3.6b), and melting curve analysis (3.6c) of two separate runs.

Figure 3.1

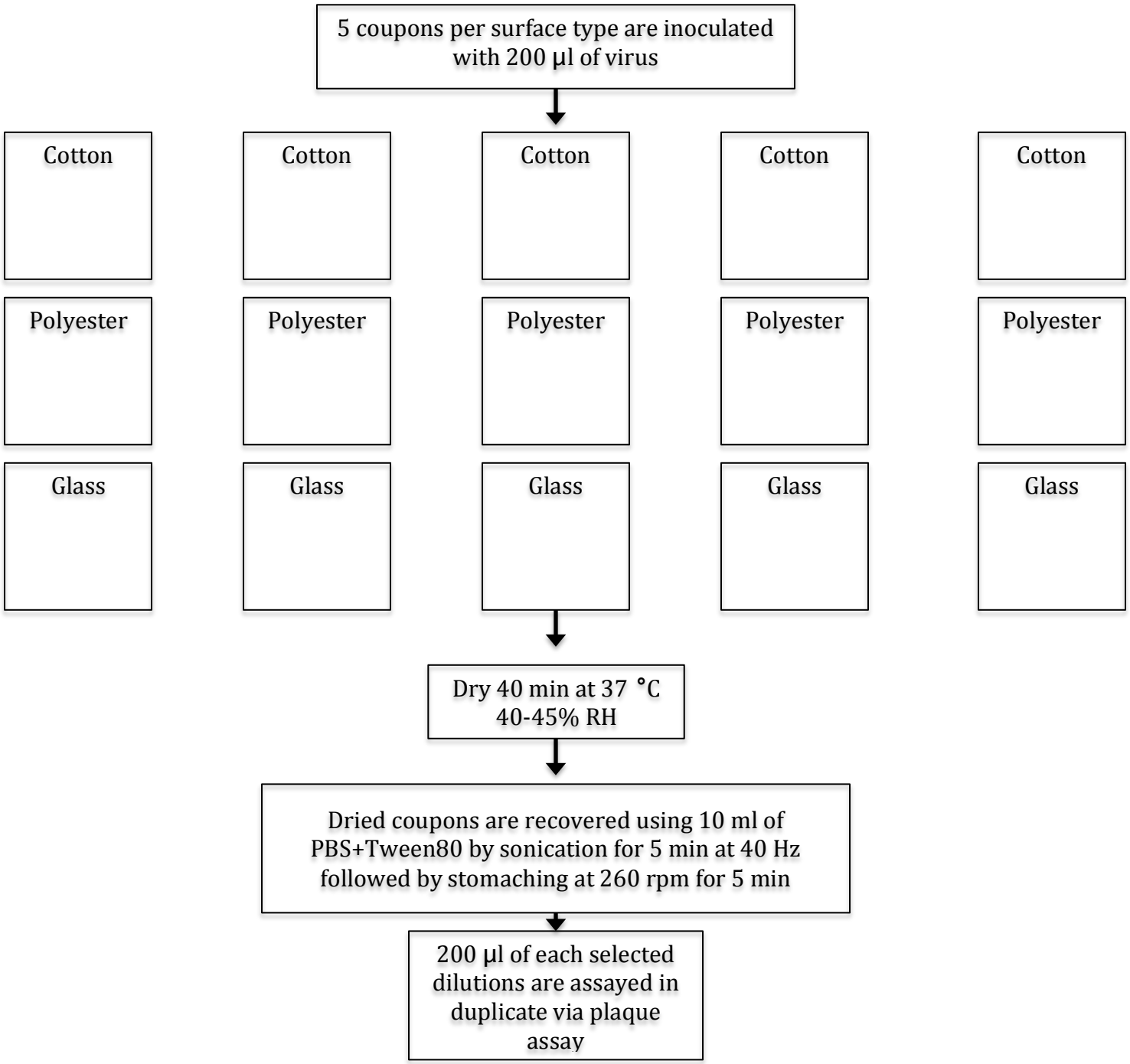

5 coupons per surface type are inoculated with 200 µl of virus
Cotton
Cotton
Cotton
Cotton
Cotton
Polyester
Polyester
Polyester
Polyester
Polyester
Glass
Glass
Glass
Glass
Glass
Dry 40 min at 37 °C 40-45% RH
Dried coupons are recovered using 10 ml of PBS+Tween80 by sonication for 5 min at 40 Hz followed by stomaching at 260 rpm for 5 min
200 µl of each selected dilutions are assayed in duplicate via plaque assay

Figure 3.2

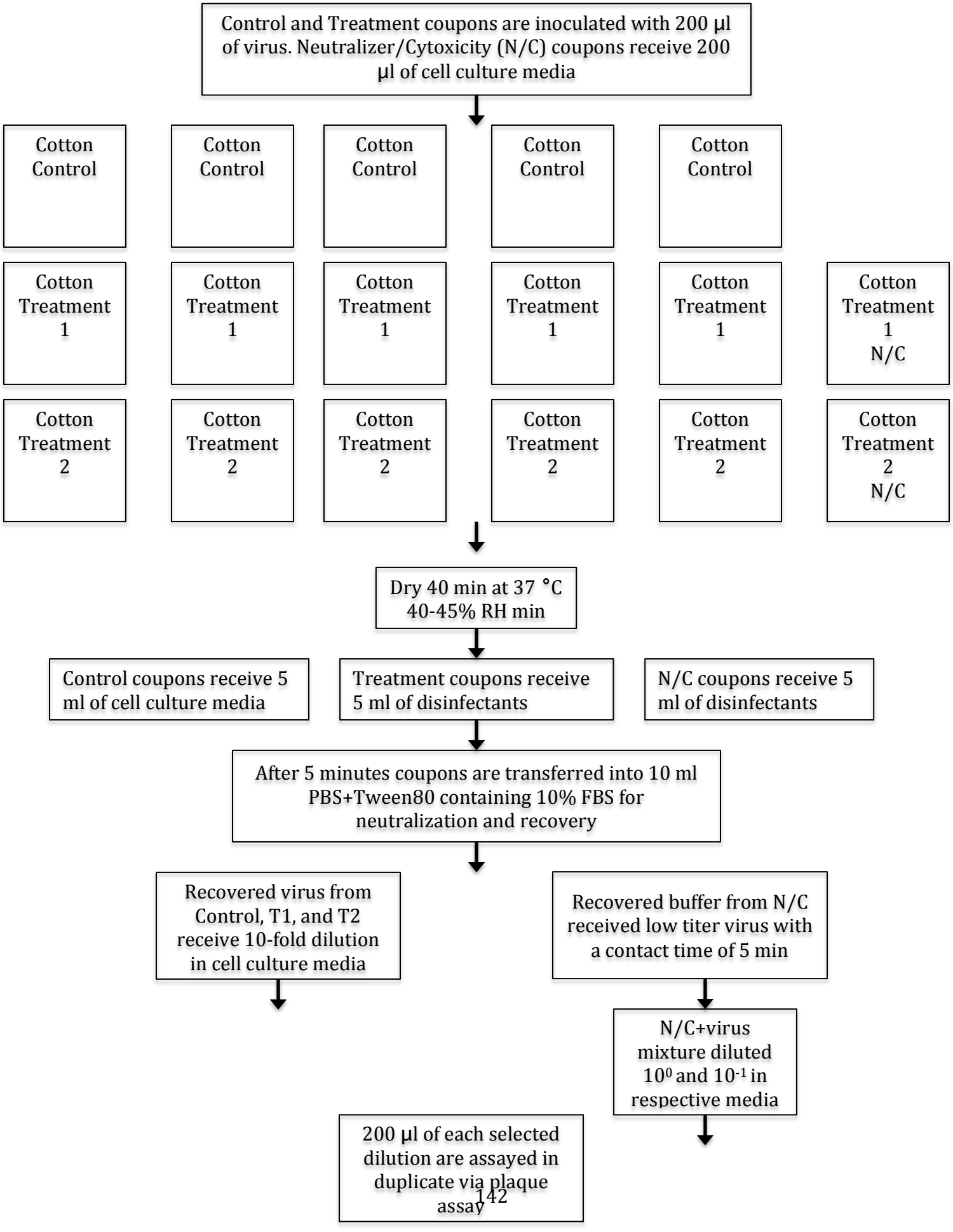

Control and Treatment coupons are inoculated with 200 µl of virus. Neutralizer/Cytoxicity (N/C) coupons receive 200 µl of cell culture media
Cotton Control
Cotton Control
Cotton Control
Cotton Control
Cotton Control
Cotton Treatment 1
Cotton Treatment 1
Cotton Treatment 1
Cotton Treatment 1
Cotton Treatment 1
Cotton Treatment 1 N/C
Cotton Treatment 2
Cotton Treatment 2
Cotton Treatment 2
Cotton Treatment 2
Cotton Treatment 2
Cotton Treatment 2 N/C
Dry 40 min at 37 °C 40-45% RH min
Control coupons receive 5 ml of cell culture media
Treatment coupons receive 5 ml of disinfectants
N/C coupons receive 5 ml of disinfectants
After 5 minutes coupons are transferred into 10 ml PBS+Tween80 containing 10% FBS for neutralization and recovery
Recovered virus from Control, T1, and T2 receive 10-fold dilution in cell culture media
Recovered buffer from N/C received low titer virus with a contact time of 5 min
N/C+virus mixture diluted 10^0 and 10^{-1} in respective media
200 µl of each selected dilution are assayed in duplicate via plaque assay

Figure 3.3

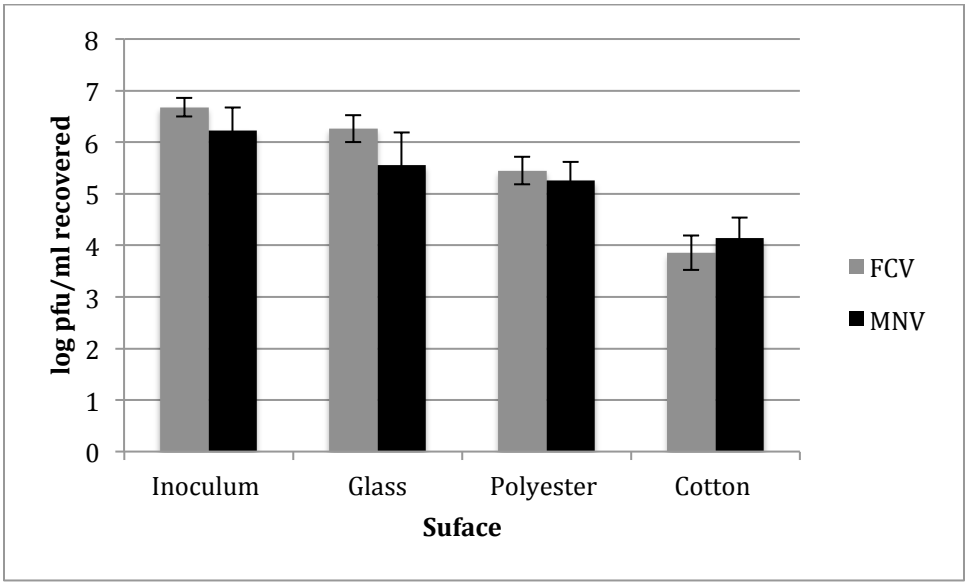


Figure 3.4

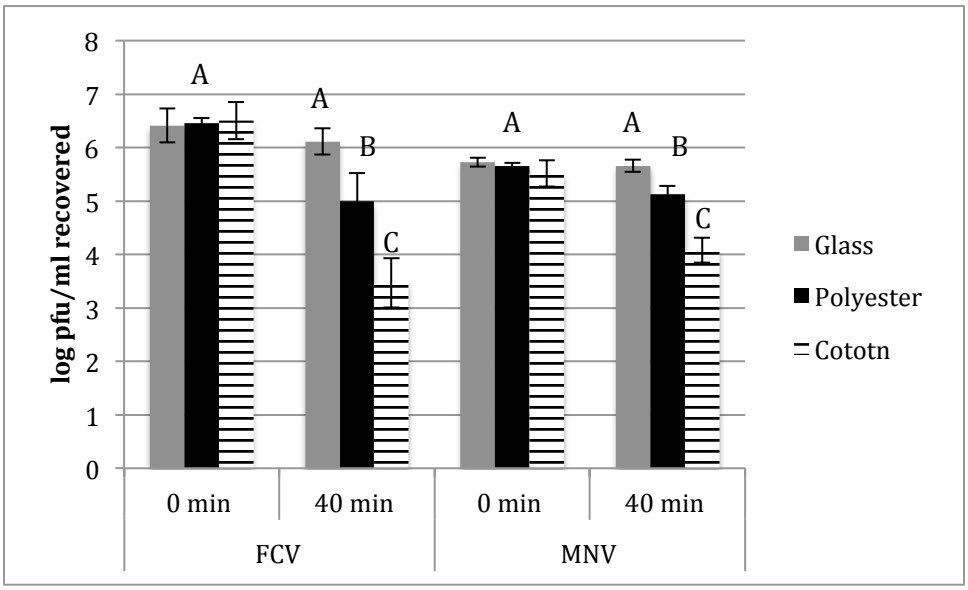
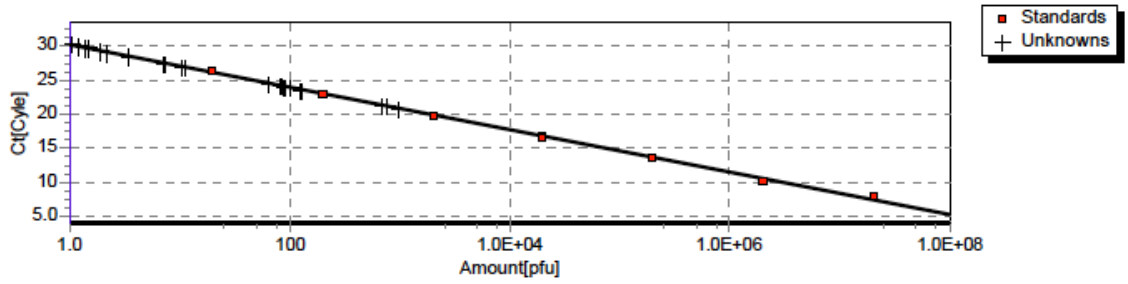
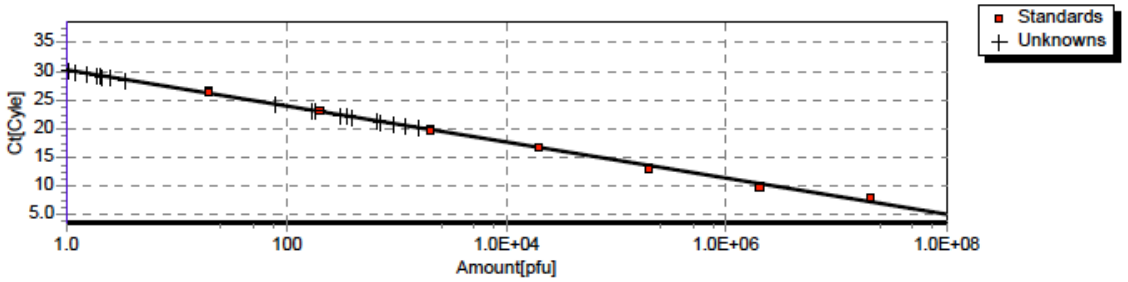


Figure 3.5a



Slope	-3.118	R^2	0.997
Y-Intercept	30.18	Efficiency	1.09



Slope	-3.155	R^2	0.996
Y-Intercept	30.31	Efficiency	1.07

Figure 3.5b

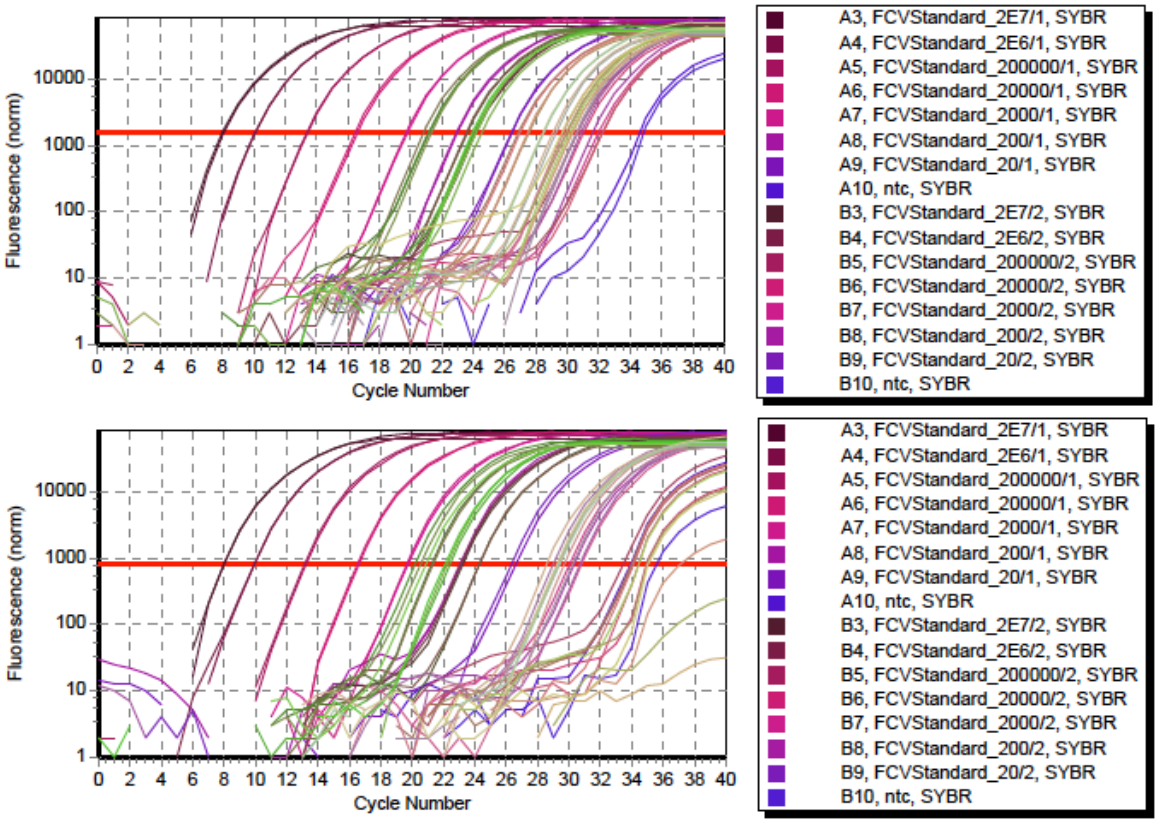

Fluorescence (norm)
10000
1000
100
10
1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle Number
A3, FCVStandard_2E7/1, SYBR
A4, FCVStandard_2E6/1, SYBR
A5, FCVStandard_200000/1, SYBR
A6, FCVStandard_20000/1, SYBR
A7, FCVStandard_2000/1, SYBR
A8, FCVStandard_200/1, SYBR
A9, FCVStandard_20/1, SYBR
A10, ntc, SYBR
B3, FCVStandard_2E7/2, SYBR
B4, FCVStandard_2E6/2, SYBR
B5, FCVStandard_200000/2, SYBR
B6, FCVStandard_20000/2, SYBR
B7, FCVStandard_2000/2, SYBR
B8, FCVStandard_200/2, SYBR
B9, FCVStandard_20/2, SYBR
B10, ntc, SYBR
Fluorescence (norm)
10000
1000
100
10
1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle Number
A3, FCVStandard_2E7/1, SYBR
A4, FCVStandard_2E6/1, SYBR
A5, FCVStandard_200000/1, SYBR
A6, FCVStandard_20000/1, SYBR
A7, FCVStandard_2000/1, SYBR
A8, FCVStandard_200/1, SYBR
A9, FCVStandard_20/1, SYBR
A10, ntc, SYBR
B3, FCVStandard_2E7/2, SYBR
B4, FCVStandard_2E6/2, SYBR
B5, FCVStandard_200000/2, SYBR
B6, FCVStandard_20000/2, SYBR
B7, FCVStandard_2000/2, SYBR
B8, FCVStandard_200/2, SYBR
B9, FCVStandard_20/2, SYBR
B10, ntc, SYBR

Figure 3.5c

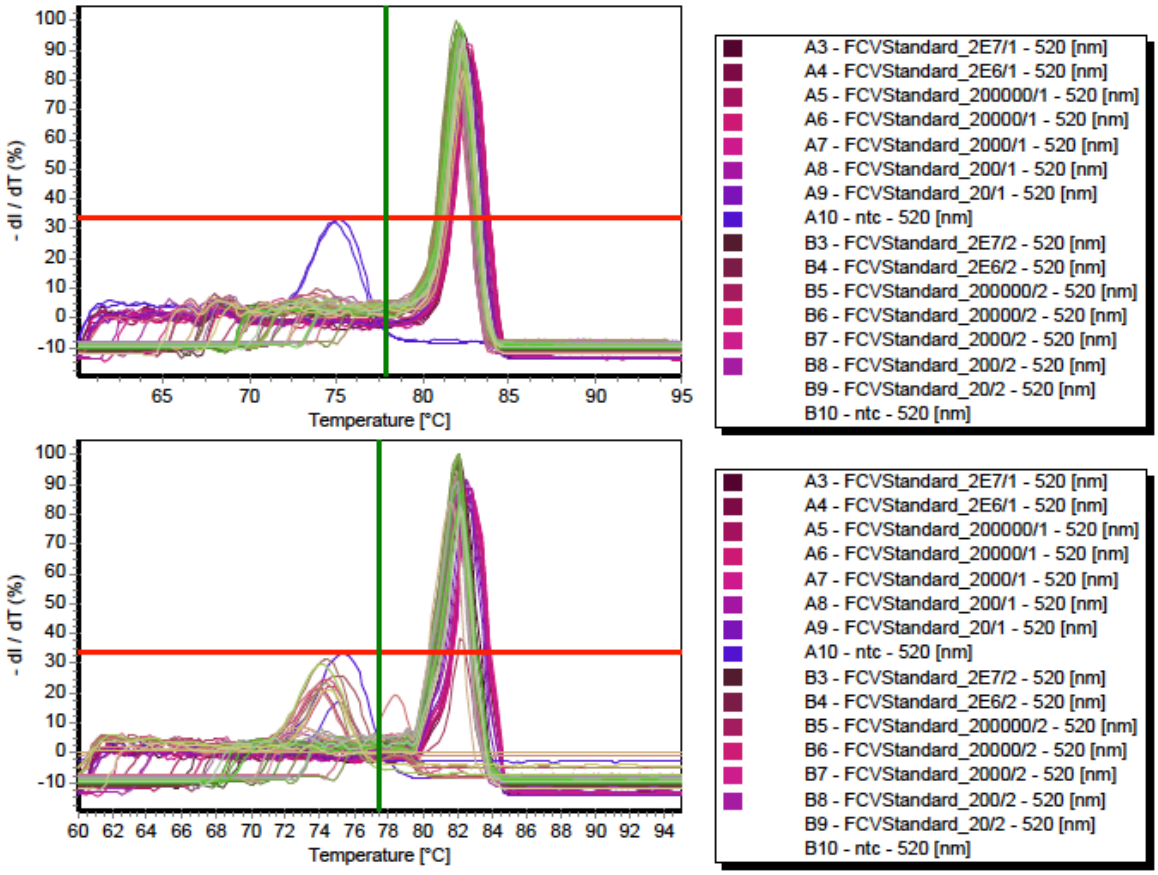
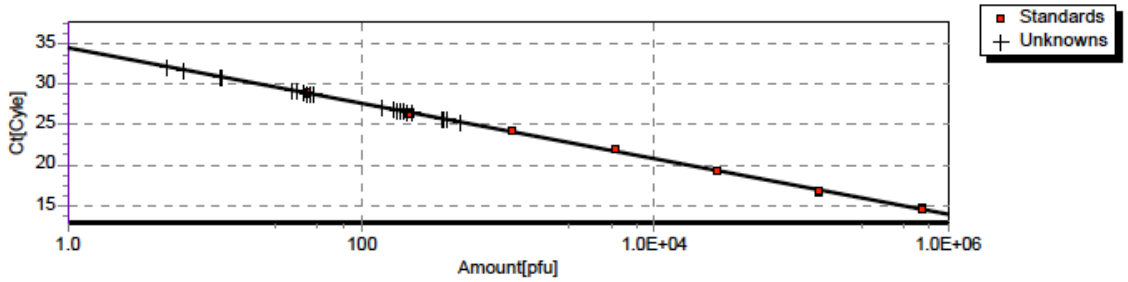
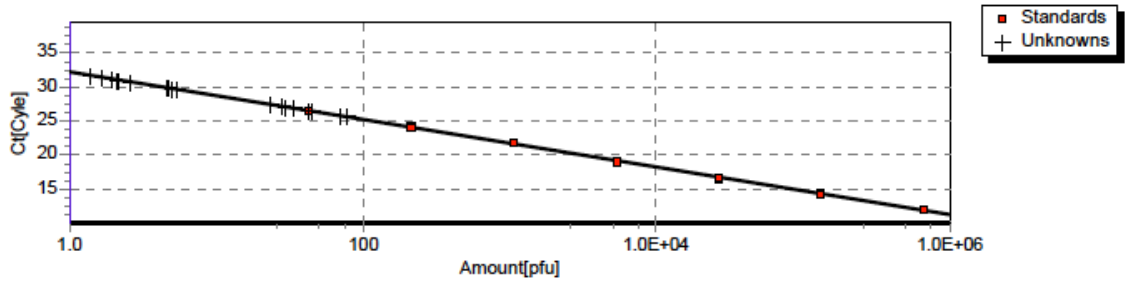

- dI / dT (%)
Temperature [°C]
A3 - FCVStandard_2E7/1 - 520 [nm]
A4 - FCVStandard_2E6/1 - 520 [nm]
A5 - FCVStandard_200000/1 - 520 [nm]
A6 - FCVStandard_20000/1 - 520 [nm]
A7 - FCVStandard_2000/1 - 520 [nm]
A8 - FCVStandard_200/1 - 520 [nm]
A9 - FCVStandard_20/1 - 520 [nm]
A10 - ntc - 520 [nm]
B3 - FCVStandard_2E7/2 - 520 [nm]
B4 - FCVStandard_2E6/2 - 520 [nm]
B5 - FCVStandard_200000/2 - 520 [nm]
B6 - FCVStandard_20000/2 - 520 [nm]
B7 - FCVStandard_2000/2 - 520 [nm]
B8 - FCVStandard_200/2 - 520 [nm]
B9 - FCVStandard_20/2 - 520 [nm]
B10 - ntc - 520 [nm]

Figure 3.6a



Slope	-3.437	R^2	1.000
Y-Intercept	34.54	Efficiency	0.95



Slope	-3.483	R^2	0.999
Y-Intercept	32.19	Efficiency	0.94

Figure 3.6b

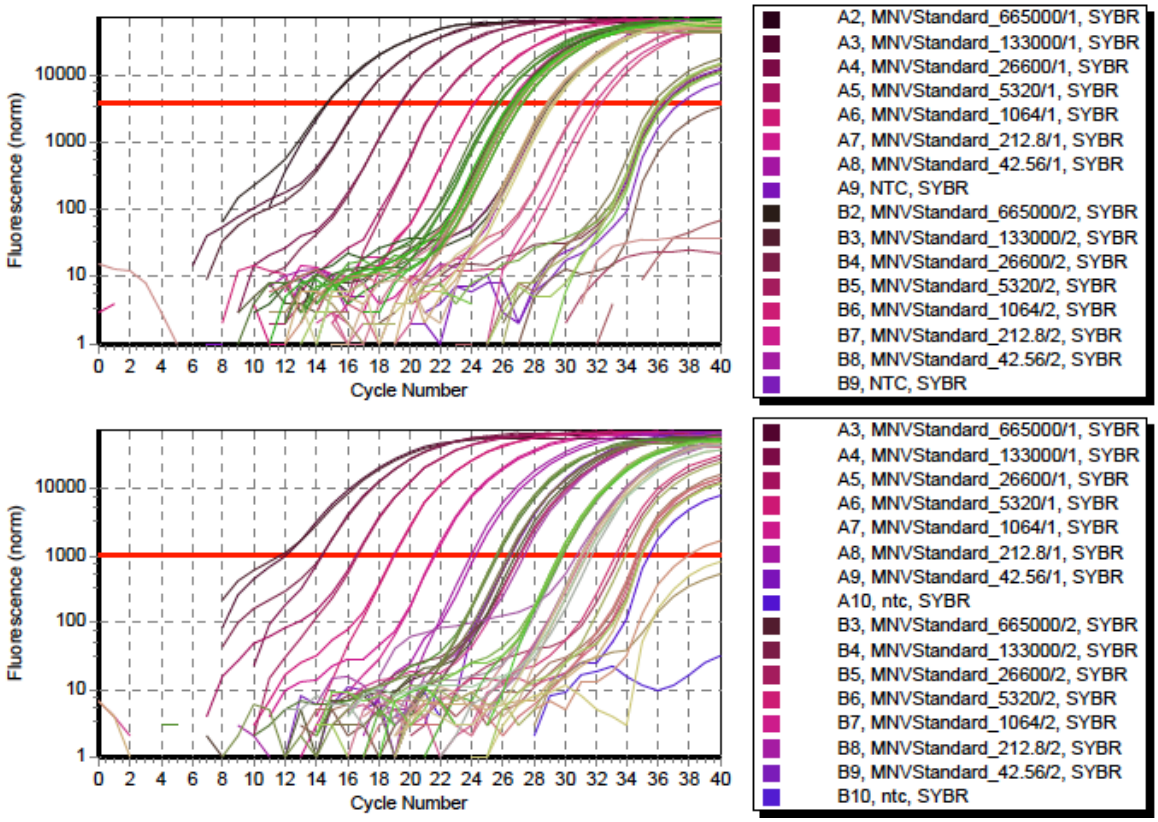

Fluorescence (norm)
10000
1000
100
10
1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle Number
A2, MNVStandard_665000/1, SYBR
A3, MNVStandard_133000/1, SYBR
A4, MNVStandard_26600/1, SYBR
A5, MNVStandard_5320/1, SYBR
A6, MNVStandard_1064/1, SYBR
A7, MNVStandard_212.8/1, SYBR
A8, MNVStandard_42.56/1, SYBR
A9, NTC, SYBR
B2, MNVStandard_665000/2, SYBR
B3, MNVStandard_133000/2, SYBR
B4, MNVStandard_26600/2, SYBR
B5, MNVStandard_5320/2, SYBR
B6, MNVStandard_1064/2, SYBR
B7, MNVStandard_212.8/2, SYBR
B8, MNVStandard_42.56/2, SYBR
B9, NTC, SYBR
Fluorescence (norm)
10000
1000
100
10
1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle Number
A3, MNVStandard_665000/1, SYBR
A4, MNVStandard_133000/1, SYBR
A5, MNVStandard_26600/1, SYBR
A6, MNVStandard_5320/1, SYBR
A7, MNVStandard_1064/1, SYBR
A8, MNVStandard_212.8/1, SYBR
A9, MNVStandard_42.56/1, SYBR
A10, ntc, SYBR
B3, MNVStandard_665000/2, SYBR
B4, MNVStandard_133000/2, SYBR
B5, MNVStandard_26600/2, SYBR
B6, MNVStandard_5320/2, SYBR
B7, MNVStandard_1064/2, SYBR
B8, MNVStandard_212.8/2, SYBR
B9, MNVStandard_42.56/2, SYBR
B10, ntc, SYBR

Figure 3.6c

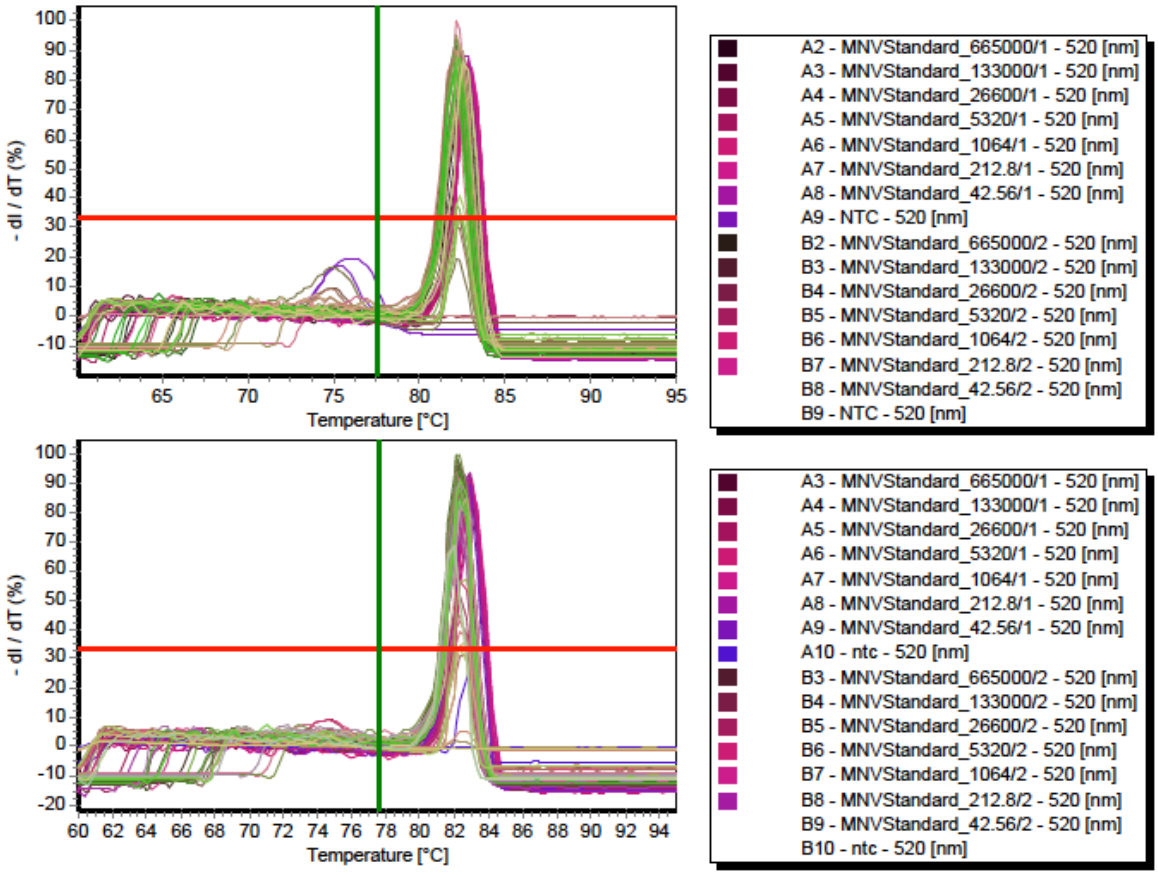

- dI / dT (%)
Temperature [°C]
A2 - MNVStandard_665000/1 - 520 [nm]
A3 - MNVStandard_133000/1 - 520 [nm]
A4 - MNVStandard_26600/1 - 520 [nm]
A5 - MNVStandard_5320/1 - 520 [nm]
A6 - MNVStandard_1064/1 - 520 [nm]
A7 - MNVStandard_212.8/1 - 520 [nm]
A8 - MNVStandard_42.56/1 - 520 [nm]
A9 - NTC - 520 [nm]
B2 - MNVStandard_665000/2 - 520 [nm]
B3 - MNVStandard_133000/2 - 520 [nm]
B4 - MNVStandard_26600/2 - 520 [nm]
B5 - MNVStandard_5320/2 - 520 [nm]
B6 - MNVStandard_1064/2 - 520 [nm]
B7 - MNVStandard_212.8/2 - 520 [nm]
B8 - MNVStandard_42.56/2 - 520 [nm]
B9 - NTC - 520 [nm]
- dI / dT (%)
Temperature [°C]
A3 - MNVStandard_665000/1 - 520 [nm]
A4 - MNVStandard_133000/1 - 520 [nm]
A5 - MNVStandard_26600/1 - 520 [nm]
A6 - MNVStandard_5320/1 - 520 [nm]
A7 - MNVStandard_1064/1 - 520 [nm]
A8 - MNVStandard_212.8/1 - 520 [nm]
A9 - MNVStandard_42.56/1 - 520 [nm]
A10 - ntc - 520 [nm]
B3 - MNVStandard_665000/2 - 520 [nm]
B4 - MNVStandard_133000/2 - 520 [nm]
B5 - MNVStandard_26600/2 - 520 [nm]
B6 - MNVStandard_5320/2 - 520 [nm]
B7 - MNVStandard_1064/2 - 520 [nm]
B8 - MNVStandard_212.8/2 - 520 [nm]
B9 - MNVStandard_42.56/2 - 520 [nm]
B10 - ntc - 520 [nm]

Table 3.1 Recovery efficiency of FCV and MNV from 3 surface types

	Recovery Efficiency (%)		
	Glass	Polyester	Cotton
FCV	35.22±0.26[a]	5.59±0.26[b]	0.15±0.33[c]
MNV	24.27±0.62[a]	14.69±0.36[b]	0.85±0.39[d]

Different superscript denotes groups that are significantly different.

Table 3.2 Virucidal efficacy of disinfectants against FCV and MNV in suspension

	FCV		**MNV**	
Disinfectant[a]	log reduction (pfu/ml)	% inactivation	log reduction (pfu/ml)	% inactivation
Clorox	>5.5	>99.999	>4.3	>99.99
Oxivir	>5.5	>99.999	>4.3	>99.99

[a] Two disinfectants, Clorox and Oxivir, were tested at concentrations of 5,000 ppm and 2,656 ppm, respectively, for a contact time of 5 min.

Table 3.3 Virucidal efficacy of disinfectants against FCV and MNV on 3 surfaces determined by plaque assay

		FCV		MNV	
Disinfectant[a]	Surface	log reduction (pfu/ml)	% inactivation	log reduction (pfu/ml)	% inactivation
Clorox	Glass	>4.7	>99.99	>3.8	>99.9
	Polyester	>4.1	>99.99	>3.6	>99.9
	Cotton	>2.5	>99	>2.2	>99.9
Oxivir	Glass	>4.7	>99.99	1.37±0.04	>90
	Polyester	>4.1	>99.99	0.57±0.04	<90
	Cotton	>2.5	>99	0.17±0.02	<90

[a] Two disinfectants, Clorox and Oxivir, were tested at concentrations of 5,000 ppm and 2,656 ppm, respectively, for a contact time of 5 min.

Table 3.4 Virucidal efficacy of disinfectants against FCV and MNV on 3 surfaces determined by RT-qPCR

		FCV		MNV	
Disinfectant[a]	Surface	log reduction (pfu/ml)[b]	% inactivation	log reduction (pfu/ml)[b]	% inactivation
Clorox	Glass	4.06±0.68	>99.99	2.20±0.43	>99
	Polyester	3.73±0.90	>99.9	3.04±0.50	>99.9
	Cotton	2.72+0.97	>99	2.07±0.27	>99
Oxivir	Glass	3.40±1.0	>99.9	0.85±0.59	<90
	Polyester	3.36±0.71	>99.9	0.85±0.59	<90
	Cotton	1.89±0.12	>90	0.54±0.40	<90

[a] Two disinfectants, Clorox and Oxivir, were tested at concentrations of 5,000 ppm and 2,656 ppm, respectively, for a contact time of 5 min.

[b] Log reduction of RNA for RT-qPCR was calculated by $(Ct_t-Ct_c)/k$ where Ct_t is the cycle threshold for treatment group, Ct_c is the cycle threshold for the control group, and k is the slope obtained from plotting the Ct values versus the log of the RNA copy number used for presenting the standard curve.

Table 3.5 Virucidal efficacy of various concentrations of bleach against FCV on cotton fabric

Clorox (ppm)[a]	log reduction (pfu/ml)	% inactivation
5,000	>2.5	>99
500	>2.5	>99
50	0	0

[a] Clorox was tested on cotton fabric for a contact time of 5 min.

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Conclusions

Epidemiological evidence from reported HuNoV outbreaks suggests that soft surfaces may pose a significant threat as environmental fomites. Due to the continuing occurrence of HuNoV outbreaks there is a need for standard cleaning and disinfection methods applicable to all surface types. In this study we evaluated a method to efficiently elute pathogens bound to soft surfaces using a combined method of recovery. We found that the recovery efficiency was significantly different between a non-porous (glass), synthetic porous (polyester), and natural porous (cotton) surface. In addition the recovery of viruses from cotton was significantly different due to virus type. Efficacy of disinfection was also influenced by virus type, as only one tested disinfectant was able to fully inactivate both FCV and MNV. The resistance of MNV to disinfection suggests that it may be a more suitable candidate as a surrogate for HuNoV. Though FCV was inactivated below the limit of detection by both disinfectants on all 3 surface types, the results we obtained indicate that the low recovery of viruses from soft surfaces, especially cotton, make documenting the necessary 4 log reduction in viral titer impossible. In order to identify a disinfectant that is effective against HuNoV on both hard non-porous and soft porous surfaces further study of effective recovery methods is necessary.